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## USE OF HMGB FRAGMENTS AS ANTI-INFLAMMATORY AGENTS

### RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/427,841, filed November 20, 2002, the entire teachings of which is incorporated  
5 herein by reference.

### BACKGROUND OF THE INVENTION

Inflammation is often induced by proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-6, platelet-activating factor (PAF), macrophage migration inhibitory factor (MIF), and other compounds. These  
10 proinflammatory cytokines are produced by several different cell types, most importantly immune cells (for example, monocytes, macrophages and neutrophils), but also non-immune cells such as fibroblasts, osteoblasts, smooth muscle cells, epithelial cells, and neurons. These proinflammatory cytokines contribute to various disorders during the early stages of an inflammatory cytokine cascade.

15 Inflammatory cytokine cascades contribute to deleterious characteristics, including inflammation and apoptosis, of numerous disorders. Included are disorders characterized by both localized and systemic reactions, including, without limitation, diseases involving the gastrointestinal tract and associated tissues (such as appendicitis, peptic, gastric and duodenal ulcers, peritonitis, pancreatitis, ulcerative,  
20 pseudomembranous, acute and ischemic colitis, diverticulitis, epiglottitis, achalasia, cholangitis, cholecystitis, coeliac disease, hepatitis, Crohn's disease, enteritis, and

Whipple's disease); systemic or local inflammatory diseases and conditions (such as asthma, allergy, anaphylactic shock, immune complex disease, organ ischemia, reperfusion injury, organ necrosis, hay fever, sepsis, septicemia, endotoxic shock, cachexia, hyperpyrexia, eosinophilic granuloma, granulomatosis, and sarcoidosis);

5 diseases involving the urogenital system and associated tissues (such as septic abortion, epididymitis, vaginitis, prostatitis, and urethritis); diseases involving the respiratory system and associated tissues (such as bronchitis, emphysema, rhinitis, cystic fibrosis, pneumonitis, adult respiratory distress syndrome, pneumoultramicroscopic silicovulcanoconiosis, alveolitis, bronchiolitis, pharyngitis,

10 pleurisy, and sinusitis); diseases arising from infection by various viruses (such as influenza, respiratory syncytial virus, HIV, hepatitis B virus, hepatitis C virus and herpes), bacteria (such as disseminated bacteremia, Dengue fever), fungi (such as candidiasis) and protozoal and multicellular parasites (such as malaria, filariasis, amebiasis, and hydatid cysts); dermatological diseases and conditions of the skin (such

15 as burns, dermatitis, dermatomyositis, sunburn, urticaria warts, and wheals); diseases involving the cardiovascular system and associated tissues (such as vasculitis, angitis, endocarditis, arteritis, atherosclerosis, restenosis, thrombophlebitis, pericarditis, congestive heart failure, myocarditis, myocardial ischemia, periarteritis nodosa, and rheumatic fever); diseases involving the central or peripheral nervous system and

20 associated tissues (such as Alzheimer's disease, meningitis, encephalitis, multiple sclerosis, cerebral infarction, cerebral embolism, Guillame-Barre syndrome, neuritis, neuralgia, spinal cord injury, paralysis, and uveitis); diseases of the bones, joints, muscles and connective tissues (such as the various arthritides and arthralgias, osteomyelitis, fasciitis, Paget's disease, gout, periodontal disease, rheumatoid arthritis,

25 and synovitis); other autoimmune and inflammatory disorders (such as myasthenia gravis, thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, Behcets's syndrome, allograft rejection, graft-versus-host disease, Type I diabetes, ankylosing spondylitis, Berger's disease, and Retier's syndrome); as well as various cancers, tumors

and proliferative disorders (such as Hodgkins disease); and, in any case the inflammatory or immune host response to any primary disease.

The early proinflammatory cytokines (e.g., TNF, IL-1, etc.) mediate inflammation, and induce the late release of high mobility group box 1 (HMGB1) (also  
5 known as HMG-1 and HMG1), a protein that accumulates in serum and mediates delayed lethality and further induction of early proinflammatory cytokines.

HMGB1 was first identified as the founding member of a family of DNA-binding proteins termed high mobility group box (HMGB) proteins that are critical for DNA structure and stability. It was identified nearly 40 years ago as a ubiquitously  
10 expressed nuclear protein that binds double-stranded DNA without sequence specificity.

HMGB1 binding bends DNA to promote formation and stability of nucleoprotein complexes that facilitate gene transcription of glucocorticoid receptors and RAG recombinase. The HMGB1 molecule has three domains: two DNA binding motifs termed HMGB A and HMGB B boxes, and an acidic carboxyl terminus. The  
15 two HMGB boxes are highly conserved 80 amino acid, L-shaped domains. HMGB boxes are also expressed in other transcription factors including the RNA polymerase I transcription factor human upstream-binding factor and lymphoid-specific factor.

Recent evidence has implicated HMGB1 as a cytokine mediator of delayed lethality in endotoxemia. That work demonstrated that bacterial endotoxin  
20 (lipopolysaccharide (LPS)) activates monocytes/macrophages to release HMGB1 as a late response to activation, resulting in elevated serum HMGB1 levels that are toxic. Antibodies against HMGB1 prevent lethality of endotoxin even when antibody administration is delayed until after the early cytokine response. Like other proinflammatory cytokines, HMGB1 is a potent activator of monocytes. Intratracheal  
25 application of HMGB1 causes acute lung injury, and anti-HMGB1 antibodies protect against endotoxin-induced lung edema. Serum HMGB1 levels are elevated in critically ill patients with sepsis or hemorrhagic shock, and levels are significantly higher in non-survivors as compared to survivors.

HMGB1 has also been implicated as a ligand for RAGE, a multi-ligand receptor of the immunoglobulin superfamily. RAGE is expressed on endothelial cells, smooth muscle cells, monocytes, and nerves, and ligand interaction transduces signals through MAP kinase, P21 ras, and NF- $\kappa$ B. The delayed kinetics of HMGB1 appearance during  
5 endotoxemia makes it a potentially good therapeutic target, but little is known about the molecular basis of HMGB1 signaling and toxicity.

Therefore, it would be useful to identify characteristics of HMGB1 proinflammatory activity, particularly the active domain(s) responsible for this activity, and any inhibitory effects of other domains.

## 10 SUMMARY OF THE INVENTION

The present invention is based on the discoveries that (1) the HMGB A box serves as a competitive inhibitor of HMGB proinflammatory action, and (2) the HMGB B box has the predominant proinflammatory activity of HMGB.

Accordingly, in one embodiment, the invention is a polypeptide comprising a  
15 high mobility group box protein (HMGB) A box or variant thereof, or an A box biologically active fragment or variant thereof, which can inhibit release of a proinflammatory cytokine from a cell treated with high mobility group box (HMGB) protein, wherein the HMGB A box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) A box, an HMG1L1 A box, an HMG1L4 A box, an HMGB A  
20 box polypeptide of BAC clone RP11-395A23, an HMG1L9 A box, an LOC122441 A box, an LOC139603 A box, and an HMG1L8 A box. In one embodiment, the polypeptide can be in a pharmaceutically acceptable excipient.

In another embodiment, the invention is a purified preparation of antibodies that specifically bind to a high mobility group box protein (HMGB) B box but do not  
25 specifically bind to non-B box epitopes of HMGB, wherein the antibodies can inhibit release of a proinflammatory cytokine from a cell treated with HMGB, wherein the HMGB B box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) B box, an HMG1L1 B box, an HMG1L4 B box, and an HMGB B box

polypeptide of BAC clone RP11-395A23. In one embodiment, the antibodies can be in a pharmaceutically acceptable excipient.

In still another embodiment, the invention is a polypeptide comprising a high mobility group box protein (HMGB) B box or variant thereof, or a B box biologically  
5 active fragment or variant thereof, but not comprising a full length HMGB, wherein the polypeptide can cause release of a proinflammatory cytokine from a cell, and wherein the HMGB B box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) B box, an HMG1L1 B box, an HMG1L4 B box, and an HMGB B box polypeptide of BAC clone RP11-395A23. In one embodiment, the polypeptide can be  
10 in a pharmaceutically acceptable excipient.

In other embodiment, the invention comprises vectors encoding the polypeptides described above.

In still another embodiment, the invention is a method of inhibiting release of a proinflammatory cytokine from a mammalian cell, the method comprising treating the  
15 cell with an amount of a purified preparation of antibodies that specifically bind to a high mobility group box protein (HMGB) B box but do not specifically bind to non-B box epitopes of HMGB, wherein the HMGB B box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) B box, an HMG1L1 B box, an HMG1L4 B box, and an HMGB B box polypeptide of BAC clone RP11-395A23.

20 In another embodiment, the invention is a method of inhibiting release of a proinflammatory cytokine from a mammalian cell, the method comprising treating the cell with a polypeptide comprising a high mobility group box protein (HMGB) A box or variant thereof, or an A box biologically active fragment or variant thereof, which can inhibit release of a proinflammatory cytokine from a cell treated with high mobility  
25 group box (HMGB) protein in an amount sufficient to inhibit release of the proinflammatory cytokine from the cell, wherein the HMGB A box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) A box, an HMG1L1 A box, an HMG1L4 A box, an HMGB A box polypeptide of BAC clone RP11-395A23, an HMG1L9 A box, an LOC122441 A box, an LOC139603 A box, and an HMG1L8 A

box. In one embodiment, the cell can be treated with a vector encoding a polypeptide comprising the A box polypeptide, A box biologically active fragment, or variant thereof.

In another embodiment, the invention is a method of treating a condition in a  
5 patient characterized by activation of an inflammatory cytokine cascade, comprising administering to the patient a purified preparation of antibodies that specifically bind to a high mobility group box protein (HMGB) B box but do not specifically bind to non-B box epitopes of HMGB, in an amount sufficient to inhibit the inflammatory cytokine cascade, wherein the HMGB B box is selected from the group consisting of an  
10 HMG1L5 (formerly HMG1L10) B box, an HMG1L1 B box, an HMG1L4 B box, and an HMGB B box polypeptide of BAC clone RP11-395A23.

In another embodiment, the invention is a method of treating a condition in a patient characterized by activation of an inflammatory cytokine cascade, comprising administering to the patient a polypeptide comprising a high mobility group box protein  
15 (HMGB) A box or variant thereof, or an A box biologically active fragment or variant thereof, which can inhibit release of a proinflammatory cytokine from a cell treated with high mobility group box (HMGB) protein, in an amount sufficient to inhibit release of the proinflammatory cytokine from the cell, wherein the HMGB A box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) A box, an HMG1L1 A box,  
20 an HMG1L4 A box, an HMGB A box polypeptide of BAC clone RP11-395A23, an HMG1L9 A box, an LOC122441 B box, an LOC139603 A box, and an HMG1L8 A box.

In still another embodiment, the invention is a method of stimulating the release of a proinflammatory cytokine from a cell comprising treating the cell with a  
25 polypeptide comprising a high mobility group box protein (HMGB) B box or variant thereof, or a B box biologically active fragment thereof, but not comprising a full length HMGB, in an amount sufficient to stimulate the release of the proinflammatory cytokine from the cell, wherein the HMGB B box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) B box, an HMG1L1 B box, an HMG1L4 B box, and an

HMGB B box polypeptide of BAC clone RP11-395A23. In one embodiment, the cell can be treated with a vector encoding a polypeptide comprising the B box polypeptide, B box biologically active fragment, or variant thereof.

In still another embodiment, the invention is a method for effecting weight loss or treating obesity in a patient, comprising administering to the patient an effective amount of a polypeptide comprising a high mobility group box protein (HMGB) B box or variant thereof, or a B box biologically active fragment or variant thereof, but not comprising a full length HMGB polypeptide, in an amount sufficient to stimulate the release of a proinflammatory cytokine from a cell, wherein the HMGB B box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) B box, an HMG1L1 B box, an HMG1L4 B box, and an HMGB B box polypeptide of BAC clone RP11-395A23.

In another embodiment, the invention is a method of determining whether a compound inhibits inflammation, comprising combining the compound with a) a cell that releases a proinflammatory cytokine when exposed to a high mobility group box protein (HMGB) B box or a biologically active fragment thereof; and b) the HMGB B box or biologically active fragment thereof, wherein said HMGB B box is selected from the group consisting of an HMG1L5 (formerly HMG1L10) B box, an HMG1L1 B box, an HMG1L4 B box, and an HMGB B box polypeptide of BAC clone RP11-395A23; and then determining whether the compound inhibits the release of the proinflammatory cytokine from the cell.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of HMGB1 mutants and their activity in TNF release (pg/ml).

FIG. 2A is a histogram showing the effect of 0 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml or 10 µg/ml of HMGB B box on TNF release (pg/ml) in RAW 264.7 cells.

FIG. 2B is a histogram showing the effect of 0 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml or 10 µg/ml of HMGB B box on IL-1β release (pg/ml) in RAW 264.7 cells.

FIG. 2C is a histogram showing the effect of 0  $\mu\text{g/ml}$ , 0.01  $\mu\text{g/ml}$ , 0.1  $\mu\text{g/ml}$ , 1  $\mu\text{g/ml}$  or 10  $\mu\text{g/ml}$  of HMGB B box on IL-6 release (pg/ml) in RAW 264.7 cells.

FIG. 2D a scanned image of a blot of an RNase protection assay, showing the effect of HMGB B box (at 0 hours, 4 hours, 8 hours, or 24 hours after administration) or vector alone (at 4 hours after administration) on TNF mRNA expression in RAW 264.7 cells.

FIG. 2E is a histogram of the effect of HMGB1 B box on TNF protein release (pg/ml) from RAW 264.7 cells at 0 hours, 4 hours, 8 hours, 24 hours, 32 hours or 48 hours after administration.

FIG. 2F is a histogram of the effect of vector on TNF protein release (pg/ml) from RAW 264.7 cells at 0 hours, 4 hours, 8 hours, 24 hours, 32 hours or 48 hours after administration.

FIG. 3 is a schematic representation of HMGB1 B box mutants and their activity in TNF release (pg/ml).

FIG. 4A is a graph of the effect of 0  $\mu\text{g/ml}$ , 5  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ , or 25  $\mu\text{g/ml}$  of HMGB1 A box protein on the release of TNF (as a percent of HMGB1 mediated TNF release alone) from RAW 264.7 cells.

FIG. 4B is a histogram of the effect of HMGB1 (0 or 1.5  $\mu\text{g/ml}$ ), HMGB1 A box (0 or 10  $\mu\text{g/ml}$ ), or vector (0 or 10  $\mu\text{g/ml}$ ), alone, or in combination, on the release of TNF (as a percent of HMGB1 mediated TNF release alone) from RAW 264.7 cells.

FIG. 5A is a graph of binding of  $^{125}\text{I}$ -HMGB1 binding to RAW 264.7 cells (CPM/well) over time (minutes).

FIG. 5B is a histogram of the binding of  $^{125}\text{I}$ -HMGB1 in the absence of unlabeled HMGB1 or HMGB1 A box for 2 hours at 4°C (Total), or in the presence of 5,000 molar excess of unlabeled HMGB1 (HMGB1) or A box (A box), measured as a percent of the total CPM/well.

FIG. 6 is a histogram of the effects of HMGB1 (HMG-1; 0  $\mu\text{g/ml}$  or 1  $\mu\text{g/ml}$ ) or HMGB1 B box (B Box; 0  $\mu\text{g/ml}$  or 10  $\mu\text{g/ml}$ ), alone or in combination with anti-B box



antibody (25 µg/ml or 100 µg/ml) or IgG (25 µg/ml or 100 µg/ml) on TNF release from RAW 264.7 cells (expressed as a percent of HMGB1 mediated TNF release alone).

FIG. 7A is a scanned image of a hematoxylin and eosin stained kidney section obtained from an untreated mouse.

5        FIG. 7B is a scanned image of a hematoxylin and eosin stained kidney section obtained from a mouse administered HMGB1 B box.

FIG. 7C is a scanned image of a hematoxylin and eosin stained myocardium section obtained from an untreated mouse.

10       FIG. 7D is a scanned image of a hematoxylin and eosin stained myocardium section obtained from a mouse administered HMGB1 B box.

FIG. 7E is a scanned image of a hematoxylin and eosin stained lung section obtained from an untreated mouse.

FIG. 7F is a scanned image of a hematoxylin and eosin stained lung section obtained from a mouse administered HMGB1 B box.

15       FIG. 7G is a scanned image of a hematoxylin and eosin stained liver section obtained from an untreated mouse.

FIG. 7H is a scanned image of a hematoxylin and eosin stained liver section obtained from a mouse administered HMGB1 B box.

20       FIG. 7I is a scanned image of a hematoxylin and eosin stained liver section (high magnification) obtained from an untreated mouse.

FIG. 7J is a scanned image of a hematoxylin and eosin stained liver section (high magnification) obtained from a mouse administered HMGB1 B box.

FIG. 8 is a graph of the level of HMGB1 (ng/ml) in mice subjected to cecal ligation and puncture (CLP) over time (hours).

25       FIG. 9 is a graph of the effect of HMGB A Box (60 µg/mouse or 600 µg/mouse) or no treatment on survival of mice over time (days) after cecal ligation and puncture (CLP).

FIG. 10A is a graph of the effect of anti-HMGB1 antibody (dark circles) or no treatment (open circles) on survival of mice over time (days) after cecal ligation and puncture (CLP).

FIG. 10B is a graph of the effect of anti-HMGB1 B box antiserum ( $\square$ ) or no treatment (\*) on the survival (days) of mice administered lipopolysaccharide (LPS).

FIG. 11A is a histogram of the effect of anti-RAGE antibody or non-immune IgG on TNF release from RAW 264.7 cells treated with HMGB1 (HMG-1), lipopolysaccharide (LPS), or HMGB1 B box (B box).

FIG. 11B is a histogram of the effect of HMGB1 (HMG-1) or HMGB1 B box (B Box) polypeptide stimulation on activation of the NF- $\kappa$ B-dependent ELAM promoter (measured by luciferase activity) in RAW 264.7 cells co-transfected with a murine MyD 88-dominant negative (+MyD 88 DN) mutant (corresponding to amino acids 146-296), or empty vector (-MyD 88 DN). Data are expressed as the ratio (fold-activation) of average luciferase values from unstimulated and stimulated cells (subtracted for background) + SD.

FIG. 12A is the amino acid sequence of a human HMG1 polypeptide (SEQ ID NO:1).

FIG. 12B is the amino acid sequence of rat and mouse HMG1 (SEQ ID NO:2).

FIG. 12C is the amino acid sequence of human HMG2 (SEQ ID NO:3).

FIG. 12D is the amino acid sequence of a human, mouse, and rat HMG1 A box polypeptide (SEQ ID NO:4).

FIG. 12E is the amino acid sequence of a human, mouse, and rat HMG1 B box polypeptide (SEQ ID NO:5).

FIG. 12F is the nucleic acid sequence of a forward primer for human HMG1 (SEQ ID NO:6).

FIG. 12G is the nucleic acid sequence of a reverse primer for human HMG1 (SEQ ID NO:7).

FIG. 12H is the nucleic acid sequence of a forward primer for the carboxy terminus mutant of human HMG1 (SEQ ID NO:8).

FIG. 12I is the nucleic acid sequence of a reverse primer for the carboxy terminus mutant of human HMG1 (SEQ ID NO:9).

FIG. 12J is the nucleic acid sequence of a forward primer for the amino terminus plus B box mutant of human HMG1 (SEQ ID NO:10).

5        FIG. 12K is the nucleic acid sequence of a reverse primer for the amino terminus plus B box mutant of human HMG1 (SEQ ID NO:11).

FIG. 12L is the nucleic acid sequence of a forward primer for a B box mutant of human HMG1 (SEQ ID NO:12).

10       FIG. 12M is the nucleic acid sequence of a reverse primer for a B box mutant of human HMG1 (SEQ ID NO:13).

FIG. 12N is the nucleic acid sequence of a forward primer for the amino terminus plus A box mutant of human HMG1 (SEQ ID NO:14).

FIG. 12O is the nucleic acid sequence of a reverse primer for the amino terminus plus A box mutant of human HMG1 (SEQ ID NO:15).

15       FIG. 13 is a sequence alignment of HMGB1 polypeptide sequences from rat (SEQ ID NO:2), mouse (SEQ ID NO:2), and human (SEQ ID NO:18).

FIG. 14A is the nucleic acid sequence of HMG1L5 (formerly HMG1L10) (SEQ ID NO: 32) encoding an HMGB polypeptide.

20       FIG. 14B is the polypeptide sequence of HMG1L5 (formerly HMG1L10) (SEQ ID NO: 24) encoding an HMGB polypeptide.

FIG. 14C is the nucleic acid sequence of HMG1L1 (SEQ ID NO: 33) encoding an HMGB polypeptide.

FIG. 14D is the polypeptide sequence of HMG1L1 (SEQ ID NO: 25) encoding an HMGB polypeptide.

25       FIG. 14E is the nucleic acid sequence of HMG1L4 (SEQ ID NO: 34) encoding an HMGB polypeptide.

FIG. 14F is the polypeptide sequence of HMG1L4 (SEQ ID NO: 26) encoding an HMGB polypeptide.

FIG. 14G is the nucleic acid sequence of the HMG polypeptide sequence of the BAC clone RP11-395A23 (SEQ ID NO: 35).

FIG. 14H is the polypeptide sequence of the HMG polypeptide sequence of the BAC clone RP11-395A23 (SEQ ID NO: 27) encoding an HMGB polypeptide.

5        FIG. 14I is the nucleic acid sequence of HMG1L9 (SEQ ID NO: 36) encoding an HMGB polypeptide.

FIG. 14J is the polypeptide sequence of HMG1L9 (SEQ ID NO: 28) encoding an HMGB polypeptide.

10       FIG. 14K is the nucleic acid sequence of LOC122441 (SEQ ID NO: 37) encoding an HMGB polypeptide.

FIG. 14L is the polypeptide sequence of LOC122441 (SEQ ID NO: 29) encoding an HMGB polypeptide.

FIG. 14M is the nucleic acid sequence of LOC139603 (SEQ ID NO: 38) encoding an HMGB polypeptide.

15       FIG. 14N is the polypeptide sequence of LOC139603 (SEQ ID NO: 30) encoding an HMGB polypeptide.

FIG. 14O is the nucleic acid sequence of HMG1L8 (SEQ ID NO: 39) encoding an HMGB polypeptide.

20       FIG. 14P is the polypeptide sequence of HMG1L8 (SEQ ID NO: 31) encoding an HMGB polypeptide.

#### DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell culture, molecular biology, microbiology, cell biology, and immunology, which are well within the skill of the art. Such techniques are fully  
25 explained in the literature. See, e.g., Sambrook et al., 1989, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press; Ausubel et al. (1995), "Short Protocols in Molecular Biology", John Wiley and Sons; Methods in Enzymology

(several volumes); Methods in Cell Biology (several volumes), and Methods in Molecular Biology (several volumes).

The present invention is based on a series of discoveries that further elucidate various characteristics of the ability of HMGB1 to induce production of

5 proinflammatory cytokines and inflammatory cytokine cascades. Specifically, it has been discovered that the proinflammatory active domain of HMGB1 is the B box (and in particular, the first 20 amino acids of the B box), and that antibodies specific to the B box will inhibit proinflammatory cytokine release and inflammatory cytokine cascades, with results that can alleviate deleterious symptoms caused by inflammatory cytokine

10 cascades. It has also been discovered that the A box is a weak agonist of inflammatory cytokine release, and competitively inhibits the proinflammatory activity of the B box and of HMGB1.

As used herein, an "HMGB polypeptide" or an "HMGB protein" is a substantially pure, or substantially pure and isolated polypeptide, that has been

15 separated from components that naturally accompany it, or a recombinantly produced polypeptide having the same amino acid sequence, and increases inflammation, and/or increases release of a proinflammatory cytokine from a cell, and/or increases the activity of the inflammatory cytokine cascade. In one embodiment, the HMGB polypeptide has one of the above biological activities. In another embodiment, the

20 HMGB polypeptide has two of the above biological activities. In a third embodiment, the HMGB polypeptide has all three of the above biological activities.

Preferably, the HMGB polypeptide is a mammalian HMGB polypeptide, for example, a human HMGB1 polypeptide. Examples of an HMGB polypeptide include a polypeptide comprising or consisting of the sequence of SEQ ID NO:1, SEQ ID NO:2,

25 SEQ ID NO:3, or SEQ ID NO:18. Preferably, the HMGB polypeptide contains a B box DNA binding domain and/or an A box DNA binding domain, and/or an acidic carboxyl terminus as described herein. Other examples of HMGB polypeptides are described in GenBank Accession Numbers AAA64970, AAB08987, P07155, AAA20508, S29857, P09429, NP\_002119, CAA31110, S02826, U00431, X67668, NP\_005333, NM\_016957,

and J04179, the entire teachings of which are incorporated herein by reference.

Additional examples of HMGB polypeptides include, but are not limited to mammalian HMG1 ((HMGB1) as described, for example, in GenBank Accession Number U51677), HMG2 ((HMGB2) as described, for example, in GenBank Accession Number M83665),

- 5 HMG-2A ((HMGB3, HMG-4) as described, for example, in GenBank Accession Numbers NM\_005342 and NP\_005333), HMG14 (as described, for example, in GenBank Accession Number P05114), HMG17 (as described, for example, in GenBank Accession Number X13546), HMGI (as described, for example, in GenBank Accession Number L17131), and HMGY (as described, for example, in GenBank Accession  
10 Number M23618); nonmammalian HMG T1 (as described, for example, in GenBank Accession Number X02666) and HMG T2 (as described, for example, in GenBank Accession Number L32859) (rainbow trout); HMG-X (as described, for example, in GenBank Accession Number D30765) (Xenopus), HMG D (as described, for example, in GenBank Accession Number X71138) and HMG Z (as described, for example, in  
15 GenBank Accession Number X71139) (Drosophila); NHP10 protein (HMG protein homolog NHP 1) (as described, for example, in GenBank Accession Number Z48008) (yeast); non-histone chromosomal protein (as described, for example, in GenBank Accession Number O00479) (yeast); HMG 1/ 2 like protein (as described, for example, in GenBank Accession Number Z11540) (wheat, maize, soybean); upstream binding  
20 factor (UBF-1) (as described, for example, in GenBank Accession Number X53390); PMS1 protein homolog 1 (as described, for example, in GenBank Accession Number U13695); single-strand recognition protein (SSRP, structure-specific recognition protein) (as described, for example, in GenBank Accession Number M86737); the HMG homolog TDP-1 (as described, for example, in GenBank Accession Number M74017);  
25 mammalian sex-determining region Y protein (SRY, testis-determining factor) (as described, for example, in GenBank Accession Number X53772); fungal proteins: mat-1 (as described, for example, in GenBank Accession Number AB009451), ste 11 (as described, for example, in GenBank Accession Number X53431) and Mc 1; SOX 14 (as described, for example, in GenBank Accession Number AF107043), as well as SOX 1

(as described, for example, in GenBank Accession Number Y13436), SOX 2 (as described, for example, in GenBank Accession Number Z31560), SOX 3 (as described, for example, in GenBank Accession Number X71135), SOX 6 (as described, for example, in GenBank Accession Number AF309034), SOX 8 (as described, for example, in GenBank Accession Number AF226675), SOX 10 (as described, for example, in GenBank Accession Number AJ001183), SOX 12 (as described, for example, in GenBank Accession Number X73039) and SOX 21 (as described, for example, in GenBank Accession Number AF107044)); lymphoid specific factor (LEF-1) (as described, for example, in GenBank Accession Number X58636); T-cell specific transcription factor (TCF-1) (as described, for example, in GenBank Accession Number X59869); MTT1 (as described, for example, in GenBank Accession Number M62810); and SP100-HMG nuclear autoantigen (as described, for example, in GenBank Accession Number U36501).

Other examples of HMGB proteins are polypeptides encoded by HMGB nucleic acid sequences having GenBank Accession Numbers NG\_000897 (HMG1L5 (formerly HMG1L10)) (and in particular by nucleotides 150-797-1305 of NG\_000897, as shown in FIGS. 14A and 14B); AF076674 (HMG1L1) (and in particular by nucleotides 1-633 of AF076674, as shown in FIGS. 14C and 14D); AF076676 (HMG1L4) (and in particular by nucleotides 1-564 of AF076676, as shown in FIGS. 14E and 14F); AC010149 (HMG sequence from BAC clone RP11-395A23) (and in particular by nucleotides 75503-76117 of AC010149), as shown in FIGS. 14G and 14H); AF165168 (HMG1L9) (and in particular by nucleotides 729-968 of AF165168, as shown in FIGS. 14I and 14J); XM\_063129 (LOC122441) (and in particular by nucleotides 319-558 of XM\_063129, as shown in FIGS. 14K and 14L); XM\_066789 (LOC139603) (and in particular by nucleotides 1-258 of XM\_066789, as shown in FIGS. 14M and 14N); and AF165167 (HMG1L8) (and in particular by nucleotides 456-666 of AF165167, as shown in FIGS. 14O and 14P).

As used herein, an "HMGB A box", also referred to herein as an "A box", is a substantially pure, or substantially pure and isolated polypeptide, that has been separated

from components that naturally accompany it, and consists of an amino acid sequence that is less than a full length HMGB polypeptide and which has one or more of the following biological activities: inhibiting inflammation, and/or inhibiting release of a proinflammatory cytokine from a cell, and/or decreasing the activity of the inflammatory cytokine cascade. In one embodiment, the HMGB A box polypeptide has one of the above biological activities. In another embodiment, the HMGB A box polypeptide has two of the above biological activities. In a third embodiment, the HMGB A box polypeptide has all three of the above biological activities. Preferably, the HMGB A box has no more than 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the biological activity of a full length HMGB polypeptide. In one embodiment, the HMGB A box amino acid consists of the sequence of SEQ ID NO:4, SEQ ID NO:22, or SEQ ID NO:57, or the amino acid sequence in the corresponding region of an HMGB protein in a mammal.

An HMGB A box is also a recombinantly produced polypeptide having the same amino acid sequence as the A box sequences described above. Preferably, the HMGB A box is a mammalian HMGB A box, for example, a human HMG1 A box. The HMGB A box polypeptides of the present invention preferably comprise or consist of the sequence of SEQ ID NO:4, SEQ ID NO:22, or SEQ ID NO:57, or the amino acid sequence in the corresponding region of an HMGB protein in a mammal. An HMGB A box often has no more than about 85 amino acids and no fewer than about 4 amino acids. Examples of polypeptides having A box sequences within them include, but are not limited to HMGB polypeptides described herein; GenBank Accession Numbers AAA64970, AAB08987, P07155, AAA20508, S29857, P09429, NP\_002119, CAA31110, S02826, U00431, X67668, NP\_005333, NM\_016957, and J04197, mammalian HMG1 ((HMGB1) as described, for example, in GenBank Accession Number U51677), HMG2 ((HMGB2) as described, for example, in GenBank Accession Number M83665), HMG-2A ((HMGB3, HMG-4) as described, for example, in GenBank Accession Numbers NM\_005342 and NP\_005333), HMG14 (as described, for example, in GenBank Accession Number P05114), HMG17 (as described, for example, in GenBank Accession Number X13546),



HMGI (as described, for example, in GenBank Accession Number L17131), and HMGY  
 (as described, for example, in GenBank Accession Number M23618); nonmammalian  
 HMG T1 (as described, for example, in GenBank Accession Number X02666) and  
 HMG T2 (as described, for example, in GenBank Accession Number L32859) (rainbow  
 5 trout); HMG-X (as described, for example, in GenBank Accession Number D30765)  
 (Xenopus), HMG D (as described, for example, in GenBank Accession Number  
 X71138) and HMG Z (as described, for example, in GenBank Accession Number  
 X71139) (Drosophila); NHP10 protein (HMG protein homolog NHP 1) (as described,  
 for example, in GenBank Accession Number Z48008) (yeast); non-histone chromosomal  
 10 protein (as described, for example, in GenBank Accession Number O00479) (yeast);  
 HMG 1/ 2 like protein (as described, for example, in GenBank Accession Number  
 Z11540) (wheat, maize, soybean); upstream binding factor (UBF-1) (as described, for  
 example, in GenBank Accession Number X53390); PMS1 protein homolog 1 (as  
 described, for example, in GenBank Accession Number U13695); single-strand  
 15 recognition protein (SSRP, structure-specific recognition protein) (as described, for  
 example, in GenBank Accession Number M86737); the HMG homolog TDP-1 (as  
 described, for example, in GenBank Accession Number M74017); mammalian  
 sex-determining region Y protein (SRY, testis-determining factor) (as described, for  
 example, in GenBank Accession Number X53772); fungal proteins: mat-1 (as described,  
 20 for example, in GenBank Accession Number AB009451), ste 11 (as described, for  
 example, in GenBank Accession Number X53431) and Mc 1; SOX 14 (as described, for  
 example, in GenBank Accession Number AF107043), as well as SOX 1 (as described,  
 for example, in GenBank Accession Number Y13436), SOX 2 (as described, for  
 example, in GenBank Accession Number Z31560), SOX 3 (as described, for example, in  
 25 GenBank Accession Number X71135), SOX 6 (as described, for example, in GenBank  
 Accession Number AF309034), SOX 8 (as described, for example, in GenBank  
 Accession Number AF226675), SOX 10 (as described, for example, in GenBank  
 Accession Number AJ001183), SOX 12 (as described, for example, in GenBank  
 Accession Number X73039) and SOX 21 (as described, for example, in GenBank

Accession Number AF107044)); lymphoid specific factor (LEF-1) (as described, for example, in GenBank Accession Number X58636); T-cell specific transcription factor (TCF-1) (as described, for example, in GenBank Accession Number X59869); MTT1 (as described, for example, in GenBank Accession Number M62810) and SP100-HMG  
 5 nuclear autoantigen (as described, for example, in GenBank Accession Number U36501).

Other examples of polypeptides having A box sequences within them include, but are not limited to polypeptides encoded by GenBank Accession Numbers NG\_000897 (HMG1L5 (formerly HMG1L10)) (and in particular by nucleotides 150-797  
 10 of NG\_000897, as shown in FIGS. 14A and 14B); AF076674 (HMG1L1) (and in particular by nucleotides 1-633 of AF076674, as shown in FIGS. 14C and 14D; AF076676 (HMG1L4) (and in particular by nucleotides 1-564 of AF076676, as shown in FIGS. 14E and 14F); AC010149 (HMG sequence from BAC clone RP11-395A23) (and in particular by nucleotides 75503-76117 of AC010149), as shown in FIGS. 14G  
 15 and 14H); AF165168 (HMG1L9) (and in particular by nucleotides 729-968 of AF165168, as shown in FIGS. 14I and 14J); XM\_063129 (LOC122441) (and in particular by nucleotides 319-558 of XM\_063129, as shown in FIGS. 14K and 14L); XM\_066789 (LOC139603) (and in particular by nucleotides 1-258 of XM\_066789, as shown in FIGS. 14M and 14N); and AF165167 (HMG1L8) (and in particular by  
 20 nucleotides 456-666 of AF165167, as shown in FIGS. 14O and 14P). The A box sequences in such polypeptides can be determined and isolated using methods described herein, for example, by sequence comparisons to A boxes described herein and testing for biological activity using method described herein or other methods known in the art.

Examples of HMGB A box polypeptide sequences include the following  
 25 sequences: PDASVNFSEF SKKCSERWKT MSAKEKGKFE DMAKADKARY EREMPTYIPP KGET (human HMGB1; SEQ ID NO: 40); DSSVNFAEF SKKCSERWKT MSAKEKSKFE DMAKSDKARY DREMKNYVPP KGDK (human HMGB2; SEQ ID NO: 41); PEVPVNFAEF SKKCSERWKT VSGKEKSKFD EMAKADKVRV DREMKDYGPA KGGK (human HMGB3; SEQ ID NO: 42);

- PDASVNFSEF SKKCSERWKT MSAKEKGKFE DMAKADKARY EREMPTYIPP  
KGET (HMG1L5 (formerly HMG1L10); SEQ ID NO: 43); SDASVNFSEF  
SNKCSERWKT MSAKEKGKFE DMAKADKTHY ERQMPTYIPP KGET  
(HMG1L1; SEQ ID NO: 44); PDASVNFSEF SKKCSERWKA MSAKDKGKFE  
5 DMAKVADKADY EREMPTYIPP KGET (HMG1L4; SEQ ID NO: 45); PDASVKFSEF  
LKKCSETWKT IFAKEKGKFE DMAKADKAHY EREMPTYIPP KGEK (HMG  
sequence from BAC clone RP11-395A23; SEQ ID NO: 46); PDASINFSEF  
SQKCPETWKT TIAKEKGKFE DMAKADKAHY EREMPTYIPP KGET (HMG1L9;  
SEQ ID NO: 47); PDASVNSSEF SKKCSERWKT MPTKQGKFE DMAKADRAH  
10 (HMG1L8; SEQ ID NO: 48); PDASVNFSEF SKKCLVRGKT MSAKEKGQFE  
AMARADKARY EREMPTYIP PKGET (LOC122441; SEQ ID NO: 49);  
LDASVSFSEF SNKCSERWKT MSVKEKGKFE DMAKADKACY EREMKIYPYL  
KGRQ (LOC139603; SEQ ID NO: 50); and GKGDPPKPRG KMSSYAFFVQ  
TCREEHKKKH PDASVNFSEF SKKCSERWKT MSAKEKGKFE DMAKADKARY  
15 EREMPTYIPP KGET (human HMGB1 A box; SEQ ID NO: 57).

The HMGB A box polypeptides of the present invention also encompass  
sequence variants. Variants include a substantially homologous polypeptide encoded by  
the same genetic locus in an organism, i.e., an allelic variant, as well as other variants.  
Variants also encompass polypeptides derived from other genetic loci in an organism,  
20 but having substantial homology to a polypeptide encoded by an HMGB A box nucleic  
acid molecule, and complements and portions thereof, or having substantial homology to  
a polypeptide encoded by a nucleic acid molecule comprising the nucleotide sequence of  
an HMGB A box nucleic acid molecule. Examples of HMGB A box nucleic acid  
molecules are known in the art and can be derived from HMGB A polypeptides as  
25 described herein. Variants also include polypeptides substantially homologous or  
identical to these polypeptides but derived from another organism, i.e., an ortholog.  
Variants also include polypeptides that are substantially homologous or identical to these  
polypeptides that are produced by chemical synthesis. Variants also include  
polypeptides that are substantially homologous or identical to these polypeptides that are

produced by recombinant methods. Preferably, an HMGB A box has at least 60%, more preferably, at least 70%, 75%, 80%, 85%, or 90%, and most preferably at least 95%, sequence identity to an HMGB A box polypeptide described herein, for example, the sequence of SEQ ID NO:4, SEQ ID NO:22, or SEQ ID NO:57, as determined using the  
5 BLAST program and parameters described herein and one of more of the biological activities of an HMGB A box, as determined using methods described herein or other method known in the art.

The present invention also features A box biologically active fragments. By an “A box fragment that has A box biological activity” or an “A box biologically active  
10 fragment” is meant a fragment of an HMGB A box that has the activity of an HMGB A box, as described herein. For example, the A box fragment can decrease release of a pro-inflammatory cytokine from a vertebrate cell, decrease inflammation, and/or decrease activity of the inflammatory cytokine cascade. A box fragments can be generated using standard molecular biology techniques and assaying the function of the  
15 fragment by determining if the fragment, when administered to a cell inhibits release of a proinflammatory cytokine from the cell, for example, using methods described herein. A box biologically active fragments can be used in the methods described herein in which full length A box polypeptides are used, for example, inhibiting release of a proinflammatory cytokine from a cell, or treating a patient having a condition  
20 characterized by activation of an inflammatory cytokine cascade.

As used herein, an “HMGB B box”, also referred to herein as a “B box”, is a substantially pure, or substantially pure and isolated polypeptide, that has been separated from components that naturally accompany it, and consists of an amino acid sequence that is less than a full length HMGB polypeptide and has one or more of the following  
25 biological activities: increasing inflammation, increasing release of a proinflammatory cytokine from a cell, and or increasing the activity of the inflammatory cytokine cascade. In one embodiment, the HMGB B box polypeptide has one of the above biological activities. In another embodiment, the HMGB B box polypeptide has two of the above biological activities. In a third embodiment, the HMGB B box polypeptide has all three

of the above biological activities. Preferably, the HMGB B box has at least 25%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the biological activity of a full length HMGB polypeptide. In another embodiment, the HMGB B box does not comprise an HMGB A box.

5           In another embodiment, the HMGB B box is a polypeptide that is about 90%, 80%, 70%, 60%, 50%, 40%, 35%, 30%, 25%, or 20%, the length of a full length HMGB1 polypeptide. In another embodiment, the HMGB B box comprises or consists of the sequence of SEQ ID NO:5, SEQ ID NO:20 or SEQ ID NO:58, or the amino acid sequence in the corresponding region of an HMGB protein in a mammal, but is still less  
10 than the full length HMGB polypeptide. An HMGB B box polypeptide is also a recombinantly produced polypeptide having the same amino acid sequence as an HMGB B box polypeptide described above. Preferably, the HMGB B box is a mammalian HMGB B box, for example, a human HMGB1 B box. An HMGB B box often has no more than about 85 amino acids and no fewer than about 4 amino acids. Examples of  
15 polypeptides having B box sequences within them include, but are not limited to HMGB polypeptides described herein; GenBank Accession Numbers AAA64970, AAB08987, P07155, AAA20508, S29857, P09429, NP\_002119, CAA31110, S02826, U00431, X67668, NP\_005333, NM\_016957, and J04197, mammalian HMG1 ((HMGB1) as described, for example, in GenBank Accession Number U51677), HMG2 ((HMGB2) as  
20 described, for example, in GenBank Accession Number M83665), HMG-2A ((HMGB3, HMG-4) as described, for example, in GenBank Accession Numbers NM\_005342 and NP\_005333), HMG14 (as described, for example, in GenBank Accession Number P05114), HMG17 (as described, for example, in GenBank Accession Number X13546), HMG1 (as described, for example, in GenBank Accession Number L17131), and HMGY  
25 (as described, for example, in GenBank Accession Number M23618); nonmammalian HMG T1 (as described, for example, in GenBank Accession Number X02666) and HMG T2 (as described, for example, in GenBank Accession Number L32859) (rainbow trout); HMG-X (as described, for example, in GenBank Accession Number D30765) (Xenopus), HMG D (as described, for example, in GenBank Accession Number

X71138) and HMG Z (as described, for example, in GenBank Accession Number X71139) (*Drosophila*); NHP10 protein (HMG protein homolog NHP 1) (as described, for example, in GenBank Accession Number Z48008) (yeast); non-histone chromosomal protein (as described, for example, in GenBank Accession Number O00479) (yeast);

5 HMG 1/ 2 like protein (as described, for example, in GenBank Accession Number Z11540) (wheat, maize, soybean); upstream binding factor (UBF-1) (as described, for example, in GenBank Accession Number X53390); PMS1 protein homolog 1 (as described, for example, in GenBank Accession Number U13695); single-strand recognition protein (SSRP, structure-specific recognition protein) (as described, for

10 example, in GenBank Accession Number M86737); the HMG homolog TDP-1 (as described, for example, in GenBank Accession Number M74017); mammalian sex-determining region Y protein (SRY, testis-determining factor) (as described, for example, in GenBank Accession Number X53772); fungal proteins: mat-1 (as described, for example, in GenBank Accession Number AB009451), ste 11 (as described, for

15 example, in GenBank Accession Number X53431) and Mc 1; SOX 14 (as described, for example, in GenBank Accession Number AF107043), as well as SOX 1 (as described, for example, in GenBank Accession Number Y13436), SOX 2 (as described, for example, in GenBank Accession Number Z31560), SOX 3 (as described, for example, in GenBank Accession Number X71135), SOX 6 (as described, for example, in GenBank

20 Accession Number AF309034), SOX 8 (as described, for example, in GenBank Accession Number AF226675), SOX 10 (as described, for example, in GenBank Accession Number AJ001183), SOX 12 (as described, for example, in GenBank Accession Number X73039) and SOX 21 (as described, for example, in GenBank Accession Number AF107044)); lymphoid specific factor (LEF-1) (as described, for

25 example, in GenBank Accession Number X58636); T-cell specific transcription factor (TCF-1) (as described, for example, in GenBank Accession Number X59869); MTT1 (as described, for example, in GenBank Accession Number M62810); and SP100-HMGB nuclear autoantigen (as described, for example, in GenBank Accession Number U36501).

Other examples of polypeptides having B box sequences within them include, but are not limited to polypeptides encoded by GenBank Accession Numbers NG\_000897 (HMG1L5 (formerly HMG1L10)) (and in particular by nucleotides 150-797 of NG\_000897, as shown in FIGS. 14A and 14B); AF076674 (HMG1L1) (and in particular by nucleotides 1-633 of AF076674, as shown in FIGS. 14C and 14D; 5 AF076676 (HMG1L4) (and in particular by nucleotides 1-564 of AF076676, as shown in FIGS. 14E and 14F); AC010149 (HMG sequence from BAC clone RP11-395A23) (and in particular by nucleotides 75503-76117 of AC010149), as shown in FIGS. 14G and 14H) The B box sequences in such polypeptides can be determined and isolated 10 using methods described herein, for example, by sequence comparisons to B boxes described herein and testing for biological activity.

Examples of HMGB B box polypeptide sequences include the following sequences: FKDPNAPKRP PSAFFLFCSE YRPKIKGEHP GLSIGDVAKK LGEMWNNTAA DDKQPYEKKA AKLKEYEKD IAAY (human HMGB1; SEQ ID 15 NO: 51); KKDPNAPKRP PSAFFLFCSE HRPKIKSEHP GLSIGDTAKK LGEMWSEQSA KDKQPYEQKA AKLKEYEKD IAAY (human HMGB2; SEQ ID NO: 52); FKDPNAPKRL PSAFFLFCSE YRPKIKGEHP GLSIGDVAKK LGEMWNNTAA DDKQPYEKKA AKLKEYEKD IAAY (HMG1L5 (formerly HMG1L10); SEQ ID NO: 53); FKDPNAPKRP PSAFFLFCSE YHPKIKGEHP 20 GLSIGDVAKK LGEMWNNTAA DDKQPGKKKA AKLKEYEKD IAAY (HMG1L1; SEQ ID NO: 54); FKDSNAPKRP PSAFFLFCSE YCPKIKGEHP GLPISDVAKK LVEMWNNTFA DDKQLCEKKA AKLKEYKKD TATY (HMG1L4; SEQ ID NO: 55); FKDPNAPKRP PSAFFLFCSE YRPKIKGEHP GLSIGDVVKK LAGMWNNTAA ADKQFYEKKA AKLKEYKKD IAAY (HMG 25 sequence from BAC clone RP11-359A23; SEQ ID NO: 56); and FKDPNAPKRP PSAFFLFCSE YRPKIKGEHP GLSIGDVAKK LGEMWNNTAA DDKQPYEKKA AKLKEYEKD IAAYRAKGKP DAAKKGVVKA EK (human HMGB1 box; SEQ ID NO: 58).

The HMGB B box polypeptides of the invention also encompasses sequence variants. Variants include a substantially homologous polypeptide encoded by the same genetic locus in an organism, i.e., an allelic variant, as well as other variants. Variants also encompass polypeptides derived from other genetic loci in an organism, but having

5 substantial homology to a polypeptide encoded by an HMGB box nucleic acid molecule, and complements and portions thereof, or having substantial homology to a polypeptide encoded by a nucleic acid molecule comprising the nucleotide sequence of an HMGB B box nucleic acid molecule. Examples of HMGB B box nucleic acid molecules are known in the art and can be derived from HMGB B box polypeptides as described

10 herein. Variants also include polypeptides substantially homologous or identical to these polypeptides but derived from another organism, i.e., an ortholog. Variants also include polypeptides that are substantially homologous or identical to these polypeptides that are produced by chemical synthesis. Variants also include polypeptides that are substantially homologous or identical to these polypeptides that are produced by

15 recombinant methods. Preferably, a non-naturally occurring HMGB B box polypeptide has at least 60%, more preferably, at least 70%, 75%, 80%, 85%, or 90%, and most preferably at least 95% sequence identity to the sequence of and HMGB B box as described herein, for example, the sequence of SEQ ID NO:5, SEQ ID NO:20, or SEQ ID NO:58, as determined using the BLAST program and parameters described herein.

20 Preferably, the HMGB B box consists of the sequence of SEQ ID NO:5, SEQ ID NO:20, or SEQ ID NO:58, or the amino acid sequence in the corresponding region of an HMGB protein in a mammal, and has one or more of the biological activities of an HMGB B box, determined using methods described herein or other methods known in the art.

In other embodiments, the present invention is directed to a polypeptide

25 comprising a vertebrate HMGB B box or a fragment thereof that has B box biological activity, or a non-naturally occurring HMGB B box but not comprising a full length HMGB polypeptide. By a "B box fragment that has B box biological activity" or a "B box biologically active fragment" is meant a fragment of an HMGB B box that has the activity of an HMGB B box. For example, the B box fragment can induce release of a



pro-inflammatory cytokine from a vertebrate cell or increase inflammation, or induce the inflammatory cytokine cascade. An example of such a B box fragment is the fragment comprising the first 20 amino acids of the HMGB1 B box (SEQ ID NO:16 or SEQ ID NO:23), as described herein. B box fragments can be generated using standard

- 5 molecular biology techniques and assaying the function of the fragment by determining if the fragment, when administered to a cell, increases release of a proinflammatory cytokine from the cell, as compared to a suitable control, for example, using methods described herein or other methods known in the art.

As used herein, a "cytokine" is a soluble protein or peptide which is naturally  
10 produced by mammalian cells and which acts *in vivo* as a humoral regulator at micro- to picomolar concentrations. Cytokines can, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. A proinflammatory cytokine is a cytokine that is capable of causing any of the following physiological reactions associated with inflammation: vasodilation, hyperemia, increased  
15 permeability of vessels with associated edema, accumulation of granulocytes and mononuclear phagocytes, or deposition of fibrin. In some cases, the proinflammatory cytokine can also cause apoptosis, such as in chronic heart failure, where TNF has been shown to stimulate cardiomyocyte apoptosis (Pulkki, Ann. Med. 29: 339-343, 1997; and Tsutsui et al., Immunol. Rev. 174:192-209, 2000).

- 20 Nonlimiting examples of proinflammatory cytokines are tumor necrosis factor (TNF), interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-18, interferon  $\gamma$ , HMG-1, platelet-activating factor (PAF), and macrophage migration inhibitory factor (MIF).

Proinflammatory cytokines are to be distinguished from anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, which are not mediators of inflammation.

- 25 In many instances, proinflammatory cytokines are produced in an inflammatory cytokine cascade, defined herein as an *in vivo* release of at least one proinflammatory cytokine in a mammal, wherein the cytokine release affects a physiological condition of the mammal. Thus, an inflammatory cytokine cascade is inhibited in embodiments of

the invention where proinflammatory cytokine release causes a deleterious physiological condition.

HMGB A boxes and HMGB B boxes, either naturally occurring or non-naturally occurring, include polypeptides that have sequence identity to the HMGB A boxes and  
5 HMGB B boxes described above. As used herein, two polypeptides (or a region of the polypeptides) are substantially homologous or identical when the amino acid sequences are at least about 60%, 70%, 75%, 80%, 85%, 90% or 95% or more homologous or identical. The percent identity of two amino acid sequences (or two nucleic acid sequences) can be determined by aligning the sequences for optimal comparison  
10 purposes (e.g., gaps can be introduced in the sequence of a first sequence). The amino acids or nucleotides at corresponding positions are then compared, and the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions x 100). In certain embodiments, the length of the HMGB polypeptide, HMGB A box  
15 polypeptide, or HMGB B box polypeptide aligned for comparison purposes is at least 30%, preferably, at least 40%, more preferably, at least 60%, and even more preferably, at least 70%, 80%, 90%, or 100% of the length of the reference sequence, for example, those sequence provided in FIGS. 12A-12E, FIGS. 14A-14P, and SEQ ID NOS: 18, 20, and 22. The actual comparison of the two sequences can be accomplished by  
20 well-known methods, for example, using a mathematical algorithm. A preferred, non-limiting example of such a mathematical algorithm is described in Karlin *et al.* (Proc. Natl. Acad. Sci. USA, 90:5873-5877, 1993). Such an algorithm is incorporated into the BLASTN and BLASTX programs (version 2.2) as described in Schaffer *et al.* (Nucleic Acids Res., 29:2994-3005, 2001). When utilizing BLAST and Gapped BLAST  
25 programs, the default parameters of the respective programs (e.g., BLASTN) can be used. See the Internet site for the National Center for Biotechnology Information (NCBI). In one embodiment, the database searched is a non-redundant (NR) database, and parameters for sequence comparison can be set at: no filters; Expect value of 10;

Word Size of 3; the Matrix is BLOSUM62; and Gap Costs have an Existence of 11 and an Extension of 1.

Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989).

- 5 Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG (Accelrys) sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 , and a gap penalty of 4 can be used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as
- 10 described in Torellis and Robotti (Comput. Appl. Biosci., 10: 3-5,1994); and FASTA described in Pearson and Lipman (Proc. Natl. Acad. Sci USA, 85: 2444-2448, 1988).

- In another embodiment, the percent identity between two amino acid sequences can be accomplished using the GAP program in the GCG software package (Accelrys, San Diego, California) using either a Blossom 63 matrix or a PAM250 matrix, and a gap
- 15 weight of 12, 10, 8, 6, or 4 and a length weight of 2, 3, or 4. In yet another embodiment, the percent identity between two nucleic acid sequences can be accomplished using the GAP program in the GCG software package (Accelrys, San Diego, California), using a gap weight of 50 and a length weight of 3.

#### A Box Polypeptides and Biologically Active Fragments Thereof

- 20 As described above, the present invention is directed to a polypeptide composition comprising a vertebrate HMGB A box, or a biologically active fragment thereof, which can inhibit release of a proinflammatory cytokine from a cell treated with HMG, or which can be used to treat a condition characterized by activation of an inflammatory cytokine cascade.

- 25 When referring to the effect of any of the compositions or methods of the invention on the release of proinflammatory cytokines, the use of the terms "inhibit" or "decrease" encompasses at least a small but measurable reduction in proinflammatory cytokine release. In preferred embodiments, the release of the proinflammatory cytokine

is inhibited by at least 20% over non-treated controls; in more preferred embodiments, the inhibition is at least 50%; in still more preferred embodiments, the inhibition is at least 70%, and in the most preferred embodiments, the inhibition is at least 80%.

Inhibition can be assessed using methods described herein or other methods known in the art. Such reductions in proinflammatory cytokine release are capable of reducing the deleterious effects of an inflammatory cytokine cascade in *in vivo* embodiments.

Because all vertebrate HMGB A boxes show a high degree of sequence conservation (see, for example, FIG. 13 for an amino acid sequence comparison of rat, mouse, and human HMGB polypeptides), it is believed that a vertebrate HMGB A box can inhibit release of a proinflammatory cytokine from a vertebrate cell treated with HMGB. Therefore, a vertebrate HMGB A box is within the scope of the invention. Preferably, the HMGB A box is a mammalian HMGB A box, for example, a mammalian HMGB1 A box, such as a human HMGB1 A box provided herein as SEQ ID NO:4, SEQ ID NO:22, or SEQ ID NO:57. Also included in the present invention are fragments of the HMGB1 A box having HMGB A box biological activity, as described herein.

It would also be recognized by the skilled artisan that non-naturally occurring HMGB A boxes (or biologically active fragments thereof) can be created without undue experimentation, which would inhibit release of a proinflammatory cytokine from a vertebrate cell treated with a vertebrate HMGB. These non-naturally occurring functional A boxes can be created by aligning amino acid sequences of HMGB A boxes from different sources, and making one or more substitutions in one of the sequences at amino acid positions where the A boxes differ. The substitutions are preferably made using the same amino acid residue that occurs in the compared A box. Alternatively, a conservative substitution is made from either of the residues.

Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. Conservatively substituted amino acids can be grouped according to the chemical properties of their side chains. For example, one grouping of amino acids includes those amino acids have neutral and hydrophobic side chains (a, v, l, i, p, w, f, and m); another grouping is those amino acids having neutral and polar side

chains (g, s, t, y, c, n, and q); another grouping is those amino acids having basic side chains (k, r, and h); another grouping is those amino acids having acidic side chains (d and e); another grouping is those amino acids having aliphatic side chains (g, a, v, l, and i); another grouping is those amino acids having aliphatic-hydroxyl side chains (s and t);

5 another grouping is those amino acids having amine-containing side chains (n, q, k, r, and h); another grouping is those amino acids having aromatic side chains (f, y, and w); and another grouping is those amino acids having sulfur-containing side chains (c and m). Preferred conservative amino acid substitutions groups are: r-k; e-d, y-f, l-m; v-i, and q-h.

10 While a conservative amino acid substitution would be expected to preserve the biological activity of an HMGB A box polypeptide, the following is one example of how non-naturally occurring A box polypeptides (variants) can be made by comparing the human HMGB1 A box (SEQ ID NO:4) with residues 32 to 85 of SEQ ID NO:3 of the human HMGB2 A box (SEQ ID NO:17).

15 HMGB1 pdasvnfsef skkcsrwkt msakekgkfe dmakadkary eremktyipp kget (SEQ ID NO:4)

HMGB2 pdssvnfaef skkcsrwkt msakekskfe dmaksdkary dremknyvpp kgdk (SEQ ID NO:17)

A non-naturally occurring HMGB A box can be created by, for example, by

20 substituting the alanine (a) residue at the third position in the HMGB1 A box with the serine (s) residue that occurs at the third position of the HMGB2 A box. The skilled artisan would know that the substitution would provide a functional non-naturally occurring A box because the s residue functions at that position in the HMGB2 A box. Alternatively, the third position of the HMGB1 A box can be substituted with any amino

25 acid that is conservative to alanine or serine, such as glycine (g), threonine (t), valine (v) or leucine (l). The skilled artisan would recognize that these conservative substitutions would be expected to result in a functional A box because A boxes are not invariant at

the third position, so a conservative substitution would provide an adequate structural substitute for an amino acid that is naturally occurring at that position.

Following the above method, a great many non-naturally occurring HMGB A boxes could be created without undue experimentation which would be expected to be functional, and the functionality of any particular non-naturally occurring HMGB A box could be predicted with adequate accuracy. In any event, the functionality of any non-naturally occurring HMGB A box could be determined without undue experimentation by simply adding it to cells along with an HMG, and determining whether the A box inhibits release of a proinflammatory cytokine by the cells, using, for example, methods described herein.

The cell from which the A box or an A box biologically active fragment will inhibit the release of HMG-induced proinflammatory cytokines can be any cell that can be induced to produce a proinflammatory cytokine. In preferred embodiments, the cell is an immune cell, for example, a macrophage, a monocyte, or a neutrophil.

Polypeptides comprising an A box or A box biologically active fragment that can inhibit the production of any single proinflammatory cytokine, now known or later discovered, are within the scope of the present invention. Preferably, the antibodies can inhibit the production of TNF, IL-1 $\beta$ , and/or IL-6. Most preferably, the antibodies can inhibit the production of any proinflammatory cytokines produced by the vertebrate cell.

The present invention is also directed to a composition comprising any of the above-described polypeptides, in a pharmaceutically acceptable excipient. In these embodiments, the composition can inhibit a condition characterized by activation of an inflammatory cytokine cascade. The condition can be one where the inflammatory cytokine cascade causes a systemic reaction, such as with endotoxic shock.

Alternatively, the condition can be mediated by a localized inflammatory cytokine cascade, as in rheumatoid arthritis. Nonlimiting examples of conditions which can be usefully treated using the present invention include those conditions enumerated in the background section of this specification. Preferably, the condition is appendicitis, peptic, gastric or duodenal ulcers, peritonitis, pancreatitis, ulcerative,

- pseudomembranous, acute or ischemic colitis, diverticulitis, epiglottitis, achalasia, cholangitis, cholecystitis, hepatitis, Crohn's disease, enteritis, Whipple's disease, asthma, allergy, anaphylactic shock, immune complex disease, organ ischemia, reperfusion injury, organ necrosis, hay fever, sepsis, septicemia, endotoxic shock,
- 5 cachexia, hyperpyrexia, eosinophilic granuloma, granulomatosis, sarcoidosis, septic abortion, epididymitis, vaginitis, prostatitis, urethritis, bronchitis, emphysema, rhinitis, cystic fibrosis, pneumonitis, pneumoultramicroscopic silico volcano coniosis, alveolitis, bronchiolitis, pharyngitis, pleurisy, sinusitis, influenza, respiratory syncytial virus infection, herpes infection, HIV infection, hepatitis B virus infection, hepatitis C virus
- 10 infection, disseminated bacteremia, Dengue fever, candidiasis, malaria, filariasis, amebiasis, hydatid cysts, burns, dermatitis, dermatomyositis, sunburn, urticaria, warts, wheals, vasculitis, angitis, endocarditis, arteritis, atherosclerosis, restenosis, thrombophlebitis, pericarditis, myocarditis, myocardial ischemia, periarteritis nodosa, rheumatic fever, Alzheimer's disease, coeliac disease, congestive heart failure, adult
- 15 respiratory distress syndrome, meningitis, encephalitis, multiple sclerosis, cerebral infarction, cerebral embolism, Guillame-Barre syndrome, neuritis, neuralgia, spinal cord injury, paralysis, uveitis, arthritides, arthralgias, osteomyelitis, fasciitis, Paget's disease, gout, periodontal disease, rheumatoid arthritis, synovitis, myasthenia gravis, thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, Behcets's syndrome, allograft
- 20 rejection, graft-versus-host disease, Type I diabetes, ankylosing spondylitis, Berger's disease, Type I diabetes, ankylosing spondylitis, Retier's syndrome, or Hodgkins disease. In more preferred embodiments, the condition is appendicitis, peptic, gastric or duodenal ulcers, peritonitis, pancreatitis, ulcerative, pseudomembranous, acute or ischemic colitis, hepatitis, Crohn's disease, asthma, allergy, anaphylactic shock, organ ischemia,
- 25 reperfusion injury, organ necrosis, hay fever, sepsis, septicemia, endotoxic shock, cachexia, septic abortion, disseminated bacteremia, burns, Alzheimer's disease, coeliac disease, congestive heart failure, adult respiratory distress syndrome, cerebral infarction, cerebral embolism, spinal cord injury, paralysis, allograft rejection or graft-versus-host disease. In the most preferred embodiments, the condition is endotoxic shock or

allograft rejection. Where the condition is allograft rejection, the composition may advantageously also include an immunosuppressant that is used to inhibit allograft rejection, such as cyclosporin.

The excipient included with the polypeptide in these compositions is chosen  
5 based on the expected route of administration of the composition in therapeutic applications. The route of administration of the composition depends on the condition to be treated. For example, intravenous injection may be preferred for treatment of a systemic disorder such as endotoxic shock, and oral administration may be preferred to treat a gastrointestinal disorder such as a gastric ulcer. The route of administration and  
10 the dosage of the composition to be administered can be determined by the skilled artisan, without undue experimentation, in conjunction with standard dose-response studies. Relevant circumstances to be considered in making such determinations include the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, and the severity of the patient's  
15 symptoms. Thus, depending on the condition, the antibody composition can be administered orally, parenterally, intranasally, vaginally, rectally, lingually, sublingually, buccally, intrabuccally and transdermally to the patient.

Accordingly, compositions designed for oral, lingual, sublingual, buccal and intrabuccal administration can be made without undue experimentation by means well  
20 known in the art, for example, with an inert diluent or with an edible carrier. The compositions may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the pharmaceutical compositions of the present invention may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like.

25       Tablets, pills, capsules, troches and the like may also contain binders, recipients, disintegrating agent, lubricants, sweetening agents, and flavoring agents. Some examples of binders include microcrystalline cellulose, gum tragacanth and gelatin. Examples of excipients include starch and lactose. Some examples of disintegrating agents include alginic acid, corn starch and the like. Examples of lubricants include



magnesium stearate and potassium stearate. An example of a glidant is colloidal silicon dioxide. Some examples of sweetening agents include sucrose, saccharin and the like. Examples of flavoring agents include peppermint, methyl salicylate, orange flavoring and the like. Materials used in preparing these various compositions should be  
5 pharmaceutically pure and non-toxic in the amounts used.

The compositions of the present invention can easily be administered parenterally such as, for example, by intravenous, intramuscular, intrathecal or subcutaneous injection. Parenteral administration can be accomplished by incorporating the antibody compositions of the present invention into a solution or suspension. Such solutions or  
10 suspensions may also include sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol and/or other synthetic solvents. Parenteral formulations may also include antibacterial agents such as, for example, benzyl alcohol and/or methyl parabens, antioxidants such as, for example, ascorbic acid and/or sodium bisulfite, and chelating agents such as EDTA. Buffers, such  
15 as acetates, citrates and phosphates, and agents for the adjustment of tonicity, such as sodium chloride and dextrose, may also be added. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Rectal administration includes administering the pharmaceutical compositions into the rectum or large intestine. This can be accomplished using suppositories or  
20 enemas. Suppository formulations can easily be made by methods known in the art. For example, suppository formulations can be prepared by heating glycerin to about 120°C, dissolving the pharmaceutical composition in the glycerin, mixing the heated glycerin after which purified water may be added, and pouring the hot mixture into a suppository mold.

25 Transdermal administration includes percutaneous absorption of the composition through the skin. Transdermal formulations include patches, ointments, creams, gels, salves and the like.

The present invention includes nasally administering to the mammal a therapeutically effective amount of the composition. As used herein, nasally

administering or nasal administration includes administering the composition to the mucous membranes of the nasal passage or nasal cavity of the patient. As used herein, pharmaceutical compositions for nasal administration of a composition include therapeutically effective amounts of the polypeptide and/or antibody prepared by well-known methods, to be administered, for example, as a nasal spray, nasal drop, suspension, gel, ointment, cream or powder. Administration of the composition may also take place using a nasal tampon or nasal sponge.

The polypeptide and/or antibody compositions described herein can also include an antagonist of an early sepsis mediator. As used herein, an early sepsis mediator is a proinflammatory cytokine that is released from cells soon (i.e., within 30-60 min.) after induction of an inflammatory cytokine cascade (e.g., exposure to LPS). Nonlimiting examples of these cytokines are TNF, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, PAF, and MIF. Also included as early sepsis mediators are receptors for these cytokines (for example, tumor necrosis factor receptor type 1) and enzymes required for production of these cytokines, for example, interleukin-1 $\beta$  converting enzyme). Antagonists of any early sepsis mediator, now known or later discovered, can be useful for these embodiments by further inhibiting an inflammatory cytokine cascade.

Nonlimiting examples of antagonists of early sepsis mediators are antisense compounds that bind to the mRNA of the early sepsis mediator, preventing its expression (see, e.g., Ojwang et al. (Biochemistry 36:6033-6045, 1997); Pampfer et al. (Biol. Reprod. 52:1316-1326, 1995); U.S. Patent No. 6,228,642; Yahata et al. (Antisense Nucleic Acid Drug Dev. 6:55-61, 1996); and Taylor et al. (Antisense Nucleic Acid Drug Dev. 8:199-205, 1998)), ribozymes that specifically cleave the mRNA of the early sepsis mediator (see, e.g., Leavitt et al. (Antisense Nucleic Acid Drug Dev. 10: 409-414, 2000); Hendrix et al. (Biochem. J. 314 (Pt. 2): 655-661, 1996)), and antibodies that bind to the early sepsis mediator and inhibit their action (see, e.g., Kam and Targan (Expert Opin. Pharmacother. 1: 615-622, 2000); Nagahira et al. (J. Immunol. Methods 222, 83-92, 1999); Lavine et al. (J. Cereb. Blood Flow Metab. 18: 52-58, 1998); and Holmes et al. (Hybridoma 19: 363-367, 2000)). Any antagonist of an early sepsis mediator, now

known or later discovered, is envisioned as within the scope of the invention. The skilled artisan can determine the amount of early sepsis mediator to use in these compositions for inhibiting any particular inflammatory cytokine cascade without undue experimentation with routine dose-response studies.

5           Other agents that can be administered with the polypeptide compositions described herein include, e.g., Vitaxin™ and other antibodies targeting  $\alpha v\beta 3$  integrin (see, e.g., U.S. Patent No. 5,753,230, PCT Publication Nos. WO 00/78815 and WO 02/070007; the entire teachings of all of which are incorporated herein by reference) and anti-IL-9 antibodies (see, e.g., PCT Publication No. WO 97/08321; the entire teachings  
10 of which are incorporated herein by reference). Additional agents that can be administered with the polypeptide compositions described herein include, e.g., B7 antagonists (e.g., CTLA4Ig, anti-CD80 antibodies, anti-CD86 antibodies), methotrexate, and/or CD40 antagonists (e.g., anti-CD40 ligand (CD40L)) (see, e.g., Saito et al., J. Immunol. 160(9):4225-31 (1998)).

#### 15 B Box Polypeptides, Biologically Active Fragments Thereof, and Antibodies Thereto

As described above, the present invention is directed to a polypeptide composition comprising a vertebrate HMGB B box, or a biologically active fragment thereof which can increase release of a proinflammatory cytokine from a vertebrate cell treated with HMGB.

20           When referring to the effect of any of the compositions or methods of the invention on the release of proinflammatory cytokines, the use of the term "increase" encompasses at least a small but measurable rise in proinflammatory cytokine release. In preferred embodiments, the release of the proinflammatory cytokine is increased by at least 1.5-fold, at least 2-fold, at least 5-fold, or at least 10-fold, over non-treated controls.  
25           Such increases in proinflammatory cytokine release are capable of increasing the effects of an inflammatory cytokine cascade in *in vivo* embodiments. Such polypeptides can also be used to induce weight loss and/or treat obesity.

Because all HMGB B boxes show a high degree of sequence conservation (see, for example, FIG. 13 for an amino acids sequence comparison of rat, mouse, and human HMGB polypeptides), it is believed that functional non-naturally occurring HMGB B boxes can be created without undue experimentation by making one or more

5 conservative amino acid substitutions, or by comparing naturally occurring vertebrate B boxes from different sources and substituting analogous amino acids, as was discussed above with respect to the creation of functional non-naturally occurring A boxes. In particularly preferred embodiments, the B box comprises SEQ ID NO:5, SEQ ID NO: 20 or SEQ ID NO:58, which are the sequences (three different lengths) of the human

10 HMGB1 B box, or, comprises the B box sequences from the polypeptides shown in FIGS. 14A-14P, or is a fragment of an HMGB B box that has B box biological activity. For example, a 20 amino acid sequence contained within SEQ ID NO: 20 contributes to the function of the B box. This 20 amino acid B-box fragment has the following amino acid sequence: fkdnpnapkrl psafflfcse (SEQ ID NO:23). Another example of an HMGB

15 B box biologically active fragment consists of amino acids 1-20 of SEQ ID NO:5 (napkrppsaf flfcseyrp; SEQ ID NO: 16).

The invention is also directed to a purified preparation of antibodies that specifically bind to a vertebrate high mobility group protein (HMG) B box, but do not specifically bind to non-B box epitopes of HMGB1. In these embodiments, the

20 antibodies can inhibit a biological activity of a B box polypeptide, for example, the release of a proinflammatory cytokine from a vertebrate cell induced by HMGB.

The term "antibody" or "purified antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that selectively binds an

25 antigen. A molecule that selectively binds to a polypeptide of the invention is a molecule that binds to that polypeptide or a fragment thereof, but does not substantially bind other molecules in a sample, e.g., a biological sample that naturally contains the polypeptide. Preferably the antibody is at least 60%, by weight, free from proteins and naturally occurring organic molecules with which it is naturally associated. More

preferably, the antibody preparation is at least 75% or 90%, and most preferably, 99%, by weight, antibody. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')<sub>2</sub> fragments that can be generated by treating the antibody with an enzyme such as pepsin.

- 5           The invention provides polyclonal and monoclonal antibodies that selectively bind to a HMGB B box polypeptide of the invention. The term "monoclonal antibody" or "monoclonal antibody composition," as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of a polypeptide of the invention. A  
10 monoclonal antibody composition thus typically displays a single binding affinity for a particular polypeptide of the invention with which it immunoreacts.

- Polyclonal antibodies can be prepared, e.g., as described herein, by immunizing a suitable subject with a desired immunogen, e.g., an HMGB B box polypeptide of the invention or fragment thereof. The antibody titer in the immunized subject can be  
15 monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired, the antibody molecules directed against the polypeptide can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction.

- 20           At an appropriate time after immunization, e.g., when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (Nature 256:495-497, 1975), the human B cell hybridoma technique (Kozbor et al., Immunol. Today 4:72, 1983), the EBV-  
25 hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985) or trioma techniques. The technology for producing hybridomas is well known (see generally Current Protocols in Immunology, Coligan et al., (eds.) John Wiley & Sons, Inc., New York, NY, 1994). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal

immunized with an immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds a particular polypeptide (e.g., a polypeptide of the invention).

Any of the many well known protocols used for fusing lymphocytes and  
5 immortalized cell lines can be applied for the purpose of generating a monoclonal antibody to a polypeptide of the invention (see, e.g., Current Protocols in Immunology, supra; Galfre et al. (Nature, 266:55052, 1977); R.H. Kenneth, in Monoclonal Antibodies: A New Dimension In Biological Analyses, Plenum Publishing Corp., New York, New York (1980); and Lerner (Yale J. Biol. Med. 54:387-402, 1981)). Moreover,  
10 the ordinarily skilled worker will appreciate that there are many variations of such methods that also would be useful.

In one alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody to an HMGB B box polypeptide of the invention can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an  
15 antibody phage display library) with the polypeptide to thereby isolate immunoglobulin library members that bind the polypeptide. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia Recombinant Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAP™ Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents  
20 particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO  
25 90/02809; Fuchs et al., Bio/Technology 9:1370-1372, 1991; Hay et al., Hum. Antibod. Hybridomas 3:81-85, 1992; Huse et al. (Science 246:1275-1281, 1989); and Griffiths et al. (EMBO J. 12:725-734, 1993).

Additionally, recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be

made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art.

In general, antibodies of the invention (e.g., a monoclonal antibody) can be used  
5 to isolate an HMGB B box polypeptide of the invention by standard techniques, such as affinity chromatography or immunoprecipitation. A polypeptide-specific antibody can facilitate the purification of natural polypeptide from cells and of recombinantly produced polypeptide expressed in host cells. Moreover, an antibody specific for an HMGB B box polypeptide of the invention can be used to detect the polypeptide (e.g., in  
10 a cellular lysate, cell supernatant, or tissue sample) in order to evaluate the abundance and pattern of expression of the polypeptide.

Because vertebrate HMGB B boxes show a high degree of sequence conservation, it is believed that a vertebrate HMGB B box can induce release of a proinflammatory cytokine from a vertebrate cell. Therefore, antibodies against a  
15 vertebrate HMGB B box are within the scope of the invention. Preferably, the HMGB B box is a mammalian HMGB B box, more preferably a mammalian HMGB1 B box, most preferably a human HMGB1 B box, provided herein as SEQ ID NO:5, SEQ ID NO:20, or SEQ ID NO:58. Antibodies can also be directed against an HMGB B box fragment that has B box biological activity.

20 Antibodies generated against the B box immunogen can be obtained by administering the B box, a B box fragment, or cells comprising the B box or B box fragment, to an animal, preferably a nonhuman, using routine protocols. The polypeptide, such as an antigenically or immunologically equivalent derivative, is used as an antigen to immunize a mouse or other animal such as a rat or chicken. The  
25 immunogen may be associated, for example, by conjugation, with an immunogenic carrier protein, for example, bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH). Alternatively, a multiple antigenic peptide comprising multiple copies of the B box or fragment, may be sufficiently antigenic to improve immunogenicity so as to obviate the need for a carrier. Bispecific antibodies, having two

antigen binding domains where each is directed against a different B box epitope, may also be produced by routine methods.

For preparation of monoclonal antibodies, any technique known in the art that provides antibodies produced by continuous cell line cultures can be used. See, e.g.,

5 Kohler and Milstein, *supra*; and Cole et al., *supra*.

Techniques for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce single chain antibodies to HMGB, the B box or fragments thereof. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies.

10 If the antibody is used therapeutically in *in vivo* applications, the antibody is preferably modified to make it less immunogenic in the individual. For example, if the individual is human the antibody is preferably "humanized"; where the complementarity determining region(s) of the antibody is transplanted into a human antibody (for example, as described in Jones et al. (Nature 321:522-525, 1986); and Tempest et al.  
15 (Biotechnology 9:266-273, 1991)).

Phage display technology can also be utilized to select antibody genes with binding activities towards the polypeptide either from repertoires of PCR amplified v-genes of lymphocytes from humans screened for possessing anti-B box antibodies or from naive libraries (McCafferty et al., Nature 348:552-554, 1990; and Marks, et al.,  
20 Biotechnology 10:779-783, 1992). The affinity of these antibodies can also be improved by chain shuffling (Clackson et al., Nature 352: 624-628, 1991).

When the antibodies are obtained that specifically bind to HMGB B box epitopes, they can then be screened, without undue experimentation, for the ability to inhibit release of a proinflammatory cytokine. Anti-HMGB B box antibodies that can  
25 inhibit the production of any single proinflammatory cytokine, and/or inhibit the release of a proinflammatory cytokine from a cell, and/or inhibit a condition characterized by activation of an inflammatory cytokine cascade, are within the scope of the present invention. Preferably, the antibodies can inhibit the production of TNF, IL-1 $\beta$ , and/or



IL-6. Most preferably, the antibodies can inhibit the production of any proinflammatory cytokines produced by the vertebrate cell.

For methods of inhibiting release of a proinflammatory cytokine from a cell or treating a condition characterized by activation of an inflammatory cytokine cascade  
5 using antibodies to the HMGB B box or a biologically active fragment thereof, the cell can be any cell that can be induced to produce a proinflammatory cytokine. In preferred embodiments, the cell is an immune cell, for example, macrophages, monocytes, or neutrophils.

In other embodiments, the present invention is directed to a composition  
10 comprising the antibody preparations described above, in a pharmaceutically acceptable excipient. In these embodiments, the compositions can inhibit a condition characterized by the activation of an inflammatory cytokine cascade. Conditions that can be treated with these compositions have been previously enumerated.

The antibody compositions described above can also include one or more of an  
15 antagonist of an early sepsis mediator, Vitaxin™ and/or other antibodies targeting  $\alpha v \beta 3$  integrin, anti-IL-9 antibodies, B7 antagonists (e.g., CTLA4Ig, anti-CD80 antibodies, anti-CD86 antibodies), methotrexate, and/or CD40 antagonists (e.g., anti-CD40 ligand (CD40L)), as previously described.

The B box polypeptides and biologically active fragments thereof described in  
20 these embodiments can be used to induce inflammatory cytokines in the appropriate isolated cells *in vitro*, or *ex vivo*, or as a treatment *in vivo*. In any of these treatments, the polypeptide or fragment can be administered by providing a DNA or RNA vector encoding the B box or B box fragment, with the appropriate control sequences operably linked to the encoded B box or B box fragment, so that the B box or B box fragment is  
25 synthesized in the treated cell or patient. *In vivo* applications include the use of the B box polypeptides or B box fragment polypeptides or vectors as a weight loss treatment. See WO 00/47104 (the entire teachings of which are incorporated herein by reference), demonstrating that treatment with HMGB1 induces weight loss. Since the HMGB B box has the activity of the HMGB protein, the B box would also be expected to induce

weight loss. HMGB B box fragments that have the function of the B box would also be expected to induce weight loss.

In further embodiments, the present invention is also directed to a method of inhibiting the release of a proinflammatory cytokine from a mammalian cell. The  
5 method comprises treating the cell with any of the HMGB A box compositions or any of the HMGB B box or HMGB B box biologically active fragment antibody compositions discussed above.

It is believed that this method would be useful for inhibiting the cytokine release from any mammalian cell that produces a proinflammatory cytokine. However, in  
10 preferred embodiments, the cell is a macrophage, because macrophage production of proinflammatory cytokines is associated with several important diseases.

It is believed that this method is useful for the inhibition of any proinflammatory cytokine produced by mammalian cells. In preferred embodiments, the proinflammatory cytokine is TNF, IL-1 $\alpha$ , IL-1 $\beta$ , MIF or IL-6, because those proinflammatory cytokines  
15 are particularly important mediators of disease.

The methods of these embodiments are useful for *in vitro* applications, such as in studies for determining biological characteristics of proinflammatory cytokine production in cells. However, the preferred embodiments are *in vivo* therapeutic applications, where the cells are in a patient suffering from, or at risk for, a condition  
20 characterized by activation of an inflammatory cytokine cascade.

These *in vivo* embodiments are believed to be useful for any condition that is mediated by an inflammatory cytokine cascade, including any of those that have been previously enumerated. Preferred conditions include appendicitis, peptic, gastric or duodenal ulcers, peritonitis, pancreatitis, ulcerative, pseudomembranous, acute or  
25 ischemic colitis, hepatitis, Crohn's disease, asthma, allergy, anaphylactic shock, organ ischemia, reperfusion injury, organ necrosis, hay fever, sepsis, septicemia, endotoxic shock, cachexia, septic abortion, disseminated bacteremia, burns, Alzheimer's disease, cerebral infarction, cerebral embolism, spinal cord injury, paralysis, allograft rejection or graft-versus-host disease. In the most preferred embodiments, the condition is endotoxic

shock or allograft rejection. Where the condition is allograft rejection, the composition may advantageously also include an immunosuppressant that is used to inhibit allograft rejection, such as cyclosporin.

These methods can also usefully include the administration of an antagonist of an  
5 early sepsis mediator, an anti- $\alpha v \beta 3$  antibody, an anti IL-9 antibody, a B7 antagonist (e.g., CTLA4Ig, an anti-CD80 antibody, an anti-CD86 antibody), methotrexate, and/or a CD40 antagonist (e.g., anti-CD40 ligand (CD40L)). The nature of these agents has been previously discussed.

In still other embodiments, the present invention is directed to a method of  
10 treating a condition in a patient characterized by activation of an inflammatory cytokine cascade. The method comprises administering to the patient any of the HMGB A box compositions (including non-naturally occurring A box polypeptides and A box biologically active fragments) or any of the HMGB B box or B box biologically active fragment antibody compositions (including non-naturally occurring B box polypeptides  
15 or biologically active fragments thereof) discussed above. This method would be expected to be useful for any condition that is mediated by an inflammatory cytokine cascade, including any of those that have been previously enumerated. As with previously described *in vivo* methods, preferred conditions include appendicitis, peptic, gastric or duodenal ulcers, peritonitis, pancreatitis, ulcerative, pseudomembranous, acute  
20 or ischemic colitis, hepatitis, Crohn's disease, asthma, allergy, anaphylactic shock, organ ischemia, reperfusion injury, organ necrosis, hay fever, sepsis, septicemia, endotoxic shock, cachexia, septic abortion, disseminated bacteremia, burns, Alzheimer's disease, cerebral infarction, cerebral embolism, spinal cord injury, paralysis, allograft rejection or graft-versus-host disease. In the most preferred embodiments, the condition is endotoxic  
25 shock or allograft rejection. Where the condition is allograft rejection, the composition may advantageously also include an immunosuppressant that is used to inhibit allograft rejection, such as cyclosporin.

These methods can also usefully include the administration of an antagonist of an early sepsis mediator, an anti- $\alpha v \beta 3$  antibody, an anti IL-9 antibody, a B7 antagonist (e.g.,

CTLA4Ig, an anti-CD80 antibody, an anti-CD86 antibody), methotrexate, and/or a CD40 antagonist (e.g., anti-CD40 ligand (CD40L)). The nature of these agents has been previously discussed.

In other embodiments, the present invention is directed to methods of stimulating  
5 the release of a proinflammatory cytokine from a cell. The method comprises treating the cell with any of the B box polypeptides or biologically active B box fragment polypeptides, for example, polypeptides that comprise or consist of the sequence of SEQ ID NO:5, SEQ ID NO:20, SEQ ID NO:58, SEQ ID NO:16, or SEQ ID NO:23, as described herein (including non-naturally occurring B box polypeptides and fragments).  
10 This method is useful for *in vitro* applications, for example, for studying the effect of proinflammatory cytokine production on the biology of the producing cell. The method is also useful for *in vivo* applications, for example, in effecting weight loss or treating obesity in a patient, as discussed herein.

Thus, in additional embodiments, the present invention is directed to a method  
15 for effecting weight loss or treating obesity in a patient. The method comprises administering to the patient an effective amount of any of the B box polypeptides or B box fragment polypeptides described herein (including non-naturally occurring B box polypeptides and fragments), in a pharmaceutically acceptable excipient.

#### Screening for Modulators of the Release of Proinflammatory Cytokines from Cells

20 The present invention is also directed to a method of determining whether a compound (test compound) inhibits inflammation and/or an inflammatory response. The method comprises combining the compound with (a) a cell that releases a proinflammatory cytokine when exposed to a vertebrate HMGB B box or a biologically active fragment thereof, and (b) the HMGB B box or a biologically active fragment  
25 thereof, and then determining whether the compound inhibits the release of the proinflammatory cytokine from the cell, as compared to a suitable control. A compound that inhibits the release of the proinflammatory cytokine in this assay is a compound that can be used to treat inflammation and/or an inflammatory response. The HMGB B box

or biologically active HMGB B box fragment can be endogenous to the cell or can be introduced into the cell using standard recombinant molecular biology techniques.

Any cell that releases a proinflammatory cytokine in response to exposure to a vertebrate HMGB B box or biologically active fragment thereof in the absence of a test compound would be expected to be useful for this invention. It is envisioned that the cell that is selected would be important in the etiology of the condition to be treated with the inhibitory compound that is being tested. For many conditions, it is expected that the preferred cell is a human macrophage.

Any method for determining whether the compound inhibits the release of the proinflammatory cytokine from the cell would be useful for these embodiments. It is envisioned that the preferred methods are the direct measurement of the proinflammatory cytokine, for example, with any of a number of commercially available ELISA assays. However, in some embodiments, the measurement of the inflammatory effect of released cytokines may be preferable, particularly when there are several proinflammatory cytokines produced by the test cell. As previously discussed, for many important disorders, the predominant proinflammatory cytokines are TNF, IL-1 $\alpha$ , IL-1 $\beta$ , MIF or IL-6; particularly TNF.

The present invention also features a method of determining whether a compound increases an inflammatory response and/or inflammation. The method comprises combining the compound (test compound) with (a) a cell that releases a proinflammatory cytokine when exposed to a vertebrate HMGB A box or a biologically active fragment thereof, and (b) the HMGB A box or biologically active fragment, and then determining whether the compound increases the release of the proinflammatory cytokine from the cell, as compared to a suitable control. A compound that increases the release of the proinflammatory cytokine in this assay is a compound that can be used to increase an inflammatory response and/or inflammation. The HMGB A box or HMGB A box biologically active fragment can be endogenous to the cell or can be introduced into the cell using standard recombinant molecular biology techniques.

Similar to the cell types useful for identifying inhibitors of inflammation described above, any cell in which release of a proinflammatory cytokine is normally inhibited in response to exposure to a vertebrate HMGB A box or a biologically active fragment thereof in the absence of any test compound would be expected to be useful for this invention. It is envisioned that the cell that is selected would be important in the etiology of the condition to be treated with the inhibitory compound that is being tested. For many conditions, it is expected that the preferred cell is a human macrophage.

Any method for determining whether the compound increases the release of the proinflammatory cytokine from the cell would be useful for these embodiments. It is envisioned that the preferred methods are the direct measurement of the proinflammatory cytokine, for example, with any of a number of commercially available ELISA assays. However, in some embodiments, the measurement of the inflammatory effect of released cytokines may be preferable, particularly when there are several proinflammatory cytokines produced by the test cell. As previously discussed, for many important disorders, the predominant proinflammatory cytokines are TNF, IL-1 $\alpha$ , IL-1 $\beta$ , MIF or IL-6; particularly TNF.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the invention will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples and claims, be considered exemplary only.

#### Example 1: Materials and Methods

##### *Cloning of HMGB1 and Production of HMGB1 Mutants*

The following methods were used to prepare clones and mutants of human HMGB1. Recombinant full length human HMGB1 (651 base pairs; GenBank Accession Number U51677) was cloned by PCR amplification from a human brain Quick-Clone cDNA preparation (Clontech, Palo Alto, CA) using the following primers; forward primer: 5' GATGGGCAAAGGAGATCCTAAG 3' (SEQ ID NO:6) and reverse primer:

5' GCGGCCGCTTATTCATCATCATCTTC 3' (SEQ ID NO:7). Human HMGB1 mutants were cloned and purified as follows. A truncated form of human HMGB1 was cloned by PCR amplification from a Human Brain Quick-Clone cDNA preparation (Clontech, Palo Alto, CA). The primers used were (forward and reverse, respectively):

- 5 Carboxy terminus mutant (557 bp): 5' GATGGGCAAAGGAGATCCTAAG 3' (SEQ ID NO:8) and 5' GCGGCCGC TCACTTGCTTTTTTCAGCCTTGAC 3' (SEQ ID NO:9).

Amino terminus+B box mutant (486 bp): 5' GAGCATAAGAAGAAGCACCCA 3' (SEQ ID NO:10) and 5' GCGGCCGC TCACTTGCTTTTTTCAGCCTTGAC 3' (SEQ ID NO:11).

- 10 B box mutant (233 bp): 5' AAGTTCAAGGATCCCAATGCAAAG 3' (SEQ ID NO:12) and 5' GCGGCCGCTCAATATGCAGCTATATCCTTTTC 3' (SEQ ID NO:13).

Amino terminus+A box mutant (261 bp): 5' GATGGGCAAAGGAGATCCTAAG 3' (SEQ ID NO: 14) and 5' TCACTTTTTTGTCTCCCCTTTGGG 3' (SEQ ID NO:15).

A stop codon was added to each mutant to ensure the accuracy of protein size.

- 15 PCR products were subcloned into pCRII-TOPO vector EcoRI sites using the TA cloning method per manufacturer's instruction (Invitrogen, Carlsbad, CA). After amplification, the PCR product was digested with EcoRI and subcloned into an expression vector with a GST tag pGEX (Pharmacia); correct orientation and positive clones were confirmed by DNA sequencing on both strands. The recombinant plasmids
- 20 were transformed into protease deficient *E. coli* strains BL21 or BL21(DE3)plysS (Novagen, Madison, WI) and fusion protein expression was induced by isopropyl-D-thiogalactopyranoside (IPTG). Recombinant proteins were obtained using affinity purification with the glutathione Sepharose resin column (Pharmacia).

The HMGB mutants generated as described above have the following amino acid sequences:

Wild type HMGB1:

MGKGDPKKPTGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKT  
 5 SAKEKGKFEDMAKADKARYEREMKTYIPPKGETKKKFKDPNAPKRLPSAFFLFC  
 SEYRPKIKGEHPGLSIGDVAKKLGEMWNNTAADDKQPYEKKA AKLKEKYEKDI  
 AAYRAKGKPDAAKKGVVKA EKSKKKKEEEDEEDEDEDEDEDEDEDEDEDEDE  
 DDDE (SEQ ID NO:18)

Carboxy terminus mutant: MGKGDPKKPTGKMSSYAFFVQTCREEHKKKHPDAS  
 10 VNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGETKK  
 KFKDPNAPKRLPSAFFLFCSEYRPKIKGEHPGLSIGDVAKKLGEMWNNTAADDK  
 QPYEKKA AKLKEKYEKDIAAYRAKGKPDAAKKGVVKA EKSK (SEQ ID NO: 19)

B Box mutant: FKDPNAPKRLPSAFFLFCSEYRPKIKGEHPGLSIGDVAKKLGEM  
 15 WNNTAADDKQPYEKKA AKLKEKYEKDIAAY (SEQ ID NO: 20)

Amino terminus + A Box mutant: MGKGDPKKPTGKMSSYAFFVQTCREEHKKK  
 HPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKG  
 ET (SEQ ID NO: 21), wherein the A box consists of the sequence PTGKMSSYAFF  
 VQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARY  
 20 EREMKTYPKGET (SEQ ID NO:22)

A polypeptide generated from a GST vector lacking HMGB1 protein was included as a control (containing a GST tag only). To inactivate the bacterial DNA that bound to the wild type HMGB1 and some of the mutants (carboxy terminus and B box), DNase I (Life Technologies), for carboxy terminus and B box mutants, or benzonase



nuclease (Novagen, Madison, WI), for wild type HMGB1, was added at about 20 units/ml bacteria lysate. Degradation of DNA was verified by ethidium bromide staining of the agarose gel containing HMGB1 proteins before and after the treatment. The protein eluates were passed over a polymyxin B column (Pierce, Rockford, IL) to  
5 remove any contaminating LPS, and dialyzed extensively against phosphate buffered saline to remove excess reduced glutathione. The preparations were then lyophilized and redissolved in sterile water before use. LPS levels were less than 60 pg/μg protein for all of the mutants and 300 pg/μg for wild type HMG-1, as measured by Limulus amebocyte lysate assay (Bio Whittaker Inc., Walkersville, MD). The integrity of protein  
10 was verified by SDS-PAGE. Recombinant rat HMGB1 (Wang et al., Science 285: 248-251, 1999) was used in some experiments since it does not have degraded fragments as observed in purified human HMGB1.

#### *Peptide Synthesis*

Peptides were synthesized and HPLC purified at Utah State University  
15 Biotechnology Center (Logan, Utah) at 90% purity. Endotoxin was not detectable in the synthetic peptide preparations as measured by Limulus assay.

#### *Cell Culture*

Murine macrophage-like RAW 264.7 cells (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium (Life Technologies, Grand Island  
20 NY) supplemented with 10% fetal bovine serum (Gemini, Catabasas, CA), penicillin and streptomycin (Life Technologies) and were used at 90% confluence in serum-free Opti-MEM I medium (Life Technologies, Grand Island, NY). Polymyxin B (Sigma, St. Louis, MO) was routinely added at 100-1,000 units/ml to neutralize the activity of any contaminating LPS as previously described; polymyxin B alone did not influence cell  
25 viability assessed with trypan blue (Wang et al., supra). Polymyxin B was not used in experiments of synthetic peptide studies.

*Measurement of TNF Release From Cells*

TNF release was measured by a standard murine fibroblast L929 (ATCC, American Type Culture Collection, Rockville, MD) cytotoxicity bioassay (Bianchi et al., Journal of Experimental Medicine 183:927-936, 1996) with the minimum detectable  
5 concentration of 30 pg/ml. Recombinant mouse TNF was obtained from R&D system Inc., (Minneapolis, MN). Murine fibroblast L929 cells (ATCC) were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini, Catabasas, CA), penicillin (50 units/ml) and streptomycin (50 µg/ml) (Life Technologies) in a humidified incubator with 5% CO<sub>2</sub>.

10      *Antibody Production*

Polyclonal antibodies against HMGB1 B box were raised in rabbits (Cocalico Biologicals, Inc., Reamstown, PA) and assayed for titer by immunoblotting. IgG was purified from anti-HMGB1 antiserum using Protein A agarose according to manufacturer's instructions (Pierce, Rockford, IL). Anti-HMGB1 B box antibodies were  
15 affinity purified using cyanogen bromide activated Sepharose beads (Cocalico Biological, Inc.). Non-immune rabbit IgG was purchased from Sigma (St. Louis, MO). Antibodies detected full length HMGB1 and B box in immunoassay, but did not cross react with TNF, IL-1 and IL-6.

*Labeling of HMGB1 with Na-<sup>125</sup>I and cell surface binding*

20      Purified HMGB1 protein (10 µg) was radiolabeled with 0.2 mCi of carrier-free <sup>125</sup>I (NEN Life Science Products Inc., Boston, MA) using Iodo-beads (Pierce, Rockford, IL) according to the manufacturer's instructions. <sup>125</sup>I-HMGB1 protein was separated from un-reacted <sup>125</sup>I by gel chromatography columns (P6 Micro Bio-Spin Chromatography Columns, Bio-Rad Laboratories, Hercules, CA) previously equilibrated  
25 with 300 mM sodium chloride, 17.5 mM sodium citrate, pH 7.0, and 0.1% bovine serum albumin (BSA). The specific activity of the eluted HMGB1 was about 2.8 x 10<sup>6</sup> cpm/µg protein. Cell surface binding studies were performed as previously described (Yang et

al., Am. J. Physiol. 275:C675-C683, 1998). RAW 264.7 cells were plated on 24-well dishes and grown to confluence. Cells were washed twice with ice-cold PBS containing 0.1% BSA and binding was carried out at 4°C for 2 hours with 0.5 ml binding buffer containing 120 mM sodium chloride, 1.2 mM magnesium sulfate, 15 mM sodium acetate, 5 mM potassium chloride, 10 mM Tris.HCl, pH 7.4, 0.2% BSA, 5mM glucose and 25,000 cpm <sup>125</sup>I-HMGB1. At the end of the incubation the supernatants were discarded and the cells were washed three times with 0.5 ml of ice-cold PBS with 0.1% BSA and lysed with 0.5 ml of 0.5 N NaOH and 0.1% SDS for 20 minutes at room temperature. The radioactivity in the lysate was then measured using a gamma counter. Specific binding was determined as total binding minus the radioactivity obtained in the presence of an excess amount of unlabeled HMGB1 or A box proteins.

#### *Animal Experiments*

TNF knock out mice were obtained from Amgen (Thousand Oaks, CA) and were on a B6x129 background. Age-matched wild-type B6x129 mice were used as a control for the studies. Mice were bred in-house at the University of Florida specific pathogen-free transgenic mouse facility (Gainesville, FL) and were used at 6-8 weeks of age.

Male 6-8 week old Balb/c and C3H/HeJ mice were purchased from Harlen Sprague-Dawley (Indianapolis, IN) and were allowed to acclimate for 7 days before use in experiments. All animals were housed in the North Shore University Hospital Animal Facility under standard temperature, and a light and dark cycle.

#### *Cecal Ligation and Puncture*

Cecal ligation and puncture (CLP) was performed as described previously (Fink and Heard, J. Surg. Res. 49:186-196, 1990; Wichmann et al., Crit. Care Med. 26:2078-2086, 1998; and Remick et al., Shock 4:89-95, 1995). Briefly, Balb/c mice were anesthetized with 75 mg/kg ketamine (Fort Dodge, Fort Dodge, Iowa) and 20 mg/kg of xylazine (Bohringer Ingelheim, St. Joseph, MO) intramuscularly. A midline incision

was performed, and the cecum was isolated. A 6-0 prolene suture ligature was placed at a level 5.0 mm from the cecal tip away from the ileocecal valve.

The ligated cecal stump was then punctured once with a 22-gauge needle, without direct extrusion of stool. The cecum was then placed back into its normal intra-  
5 abdominal position. The abdomen was then closed with a running suture of 6-0 prolene in two layers, peritoneum and fascia separately to prevent leakage of fluid. All animals were resuscitated with a normal saline solution administered sub-cutaneously at 20 ml/kg of body weight. Each mouse received a subcutaneous injection of imipenem (0.5 mg/mouse) (Primaxin, Merck & Co., Inc., West Point, PA) 30 minutes after the surgery.  
10 Animals were then allowed to recuperate. Mortality was recorded for up to 1 week after the procedure; survivors were followed for 2 weeks to ensure no late mortalities had occurred.

#### *D-galactosamine Sensitized Mice*

The D-galactosamine-sensitized model has been described previously (Galanos et al., Proc Natl. Acad. Sci. USA 76: 5939-5943, 1979; and Lehmann et al., J. Exp. Med. 165: 657-663, 1997). Mice were injected intraperitoneally with 20 mg D-galactosamine-HCL (Sigma)/mouse (in 200 µl PBS) and 0.1 or 1 mg of either HMGB1 B box or vector protein (in 200 µl PBS). Mortality was recorded daily for up to 72 hours after injection; survivors were followed for 2 weeks, and no later deaths from B box toxicity were  
20 observed.

#### *Spleen bacteria culture*

Fourteen mice received either anti-HMGB1 antibody (n=7) or control (n=7) at 24 and 30 hours after CLP, as described herein, and were euthanized for necropsy. Spleen bacteria were recovered as described previously (Villa et al., J. Endotoxin Res. 4:197-  
25 204, 1997). Spleens were removed using sterile technique and homogenized in 2 ml of PBS. After serial dilutions with PBS, the homogenate was plated as 0.15 ml aliquots on

tryptic soy agar plates (Difco, Detroit, MI) and CFU were counted after overnight incubation at 37°C.

### *Statistical Analysis*

Data are presented as mean  $\pm$  SEM unless otherwise stated. Differences between  
5 groups were determined by two-tailed Student's t-test, one-way ANOVA followed by the least significant difference test or 2 tailed Fisher's Exact Test.

### Example 2: Mapping the HMGB1 Domains for Promotion of Cytokine Activity

HMGB1 has 2 folded DNA binding domains (A and B boxes) and a negatively-charged acidic carboxyl tail. To elucidate the structural basis of HMGB1 cytokine  
10 activity, and to map the inflammatory protein domain, we expressed full length and truncated forms of HMGB1 by mutagenesis and screened the purified proteins for stimulating activity in monocyte cultures (FIG. 1). Full length HMGB1, a mutant in which the carboxy terminus was deleted, a mutant containing only the B box, and a mutant containing only the A box were generated. These mutants of human HMGB1  
15 were made by polymerase chain reaction (PCR) using specific primers as described herein, and the mutant proteins were expressed using a glutathione S-transferase (GST) gene fusion system (Pharmacia Biotech, Piscataway, NJ) in accordance with the manufacturer's instructions. Briefly, DNA fragments, made by PCR methods, were fused to GST fusion vectors and amplified in *E. coli*. The expressed HMGB1 protein  
20 and HMGB1 mutants were then isolated using a GST affinity column.

The effect of the mutants on TNF release from Murine macrophage-like RAW 264.7 cells (ATCC) was carried out as follows. RAW 264.7 cells were cultured in RPMI 1640 medium (Life Technologies, Grand Island NY) supplemented with 10% fetal bovine serum (Gemini, Catabasas, CA), penicillin and streptomycin (Life  
25 Technologies). Polymyxin (Sigma, St. Louis, MO) was added at 100 units/ml to suppress the activity of any contaminating LPS. Cells were incubated with 1  $\mu$ g/ml of full length (wild-type) HMGB1 and each HMGB1 mutant protein in Opti-MEM I

medium for 8 hours. Conditioned supernatants (containing TNF which had been released from the cells) were collected and TNF released from the cells was measured by a standard murine fibroblast L929 (ATCC) cytotoxicity bioassay (Bianchi et al., supra) with the minimum detectable concentration of 30 pg/ml. Recombinant mouse TNF was  
5 obtained from R & D Systems Inc., (Minneapolis, MN) and used as control in these experiments. The results of this study are shown in FIG. 1. Data in FIG. 1 are all presented as mean + SEM unless otherwise indicated. (N=6-10).

As shown in FIG. 1, wild-type HMGB1 and carboxyl-truncated HMGB1 significantly stimulated TNF release by monocyte cultures (murine macrophage-like  
10 RAW 264.7 cells). The B box was a potent activator of monocyte TNF release. This stimulating effect of the B box was specific, because A box only weakly activated TNF release.

### Example 3: HMGB1 B Box Protein Promotes Cytokine Activity in a Dose Dependent Manner

15 To further examine the effect of HMGB1 B box on cytokine production, varying amounts of HMGB1 B box were evaluated for the effects on TNF, IL-1 $\beta$ , and IL-6 production in murine macrophage-like RAW 264.7 cells. RAW 264.7 cells were stimulated with HMGB1 B box protein at 0-10  $\mu$ g/ml, as indicated in FIGS. 2A-2C for 8 hours. Conditioned media were harvested and measured for TNF, IL-1 $\beta$  and IL-6 levels.  
20 TNF levels were measured as described herein, and IL-1 $\beta$  and IL-6 levels were measured using the mouse IL-1 $\beta$  and IL-6 enzyme-linked immunosorbent assay (ELISA) kits (R&D System Inc., Minneapolis, MN) and N>5 for all experiments. The results of the studies are shown in FIGS. 2A-2C.

As shown in FIG. 2A, TNF release from RAW 264.7 cells increased with  
25 increased amounts of B box administered to the cells. As shown in FIG. 2B, addition of 1  $\mu$ g/ml or 10  $\mu$ g/ml of B box resulted in increased release of IL-1 $\beta$  from RAW 264.7 cells. In addition, as shown in FIG. 2C, IL-6 release from RAW 264.7 cells increased with increased amounts of B box administered to the cells.

The kinetics of B box-induced TNF release were also examined. TNF release and TNF mRNA expression were measured in RAW 264.7 cells induced by B box polypeptide or GST tag polypeptide only used as a control (vector) (10 µg/ml) for 0 to 48 hours. Supernatants were analyzed for TNF protein levels by an L929 cytotoxicity assay (N=3-5) as described herein. For mRNA measurement, cells were plated in 100 mm plates and treated in Opti-MEM I medium containing B box polypeptide or the vector alone for 0, 4, 8, or 24 hours, as indicated in FIG. 2D. The vector only sample was assayed at the 4 hour time point. Cells were scraped off the plate and total RNA was isolated using the RNeasy RNeasy spin kit in accordance with the manufacturer's instructions (Tel-Test "B", Inc., Friendswood, TX). TNF (287 bp) was measured by RNase protection assay (Ambion, Austin, TX). Equal loading and the integrity of RNA was verified by ethidium bromide staining of the RNA sample on an agarose-formaldehyde gel. The results of the RNase protection assay are shown in FIG. 2D. As shown in FIG. 2D, B box activation of monocytes occurred at the level of gene transcription, because TNF mRNA was increased significantly in monocytes exposed to B box protein (FIG. 2B). TNF mRNA expression was maximal at 4 hours and decreased at 8 and 24 hours. The vector only control (GST tag) showed no effect on TNF mRNA expression. A similar study was carried out measuring TNF protein released from RAW 264.7 cells 0, 4, 8, 24, 32 or 48 hours after administration of B box or vector only (GST tag), using the L929 cytotoxicity assay described herein. Compared to the control (medium only), B box treatment stimulated TNF protein expression (FIG. 2E) and vector alone (FIG. 2F) did not. Data are representative of three separate experiments. Together these data indicate that the HMGB1 B box domain has cytokine activity and is responsible for the cytokine stimulating activity of full length HMGB1.

In summary, the HMGB1 B box dose-dependently stimulated release of TNF, IL-1β and IL-6 from monocyte cultures (FIGS. 2A-2C), in agreement with the inflammatory activity of full length HMGB1 (Andersson et al., J. Exp. Med. 192: 565-570, 2000). In addition, these studies indicate that maximum TNF protein release occurred within 8 hours (FIG. 2E). This delayed pattern of TNF release is similar to TNF release induced

by HMGB1 itself, and is significantly later than the kinetics of TNF induced by LPS (Andersson et al., supra).

#### Example 4: The First 20 Amino Acids of the HMGB1 B Box Stimulate TNF Activity

The TNF-stimulating activity of the HMGB1 B box was further mapped. This study was carried out as follows. Fragments of the B box were generated using synthetic peptide protection techniques, as described herein. Five HMGB1 B box fragments (from SEQ ID NO:20), containing amino acids 1-20, 16-25, 30-49, 45-64, or 60-74 of the HMGB1 B box were generated, as indicated in FIG. 3. RAW 264.7 cells were treated with B box (1  $\mu$ g/ml) or a synthetic peptide fragment of the B box (10  $\mu$ g/ml), as indicated in FIG. 3, for 10 hours and TNF release in the supernatants was measured as described herein. Data shown are mean  $\pm$  SEM, (n=3 experiments, each done in duplicate and validated using 3 separate lots of synthetic peptides). As shown in FIG. 3, TNF-stimulating activity was retained by a synthetic peptide corresponding to amino acids 1-20 of the HMGB1 B box of SEQ ID NO:20 (fkdpnapkrlpsafflfcse; SEQ ID NO:23). The TNF stimulating activity of the 1-20-mer was less potent than either the full length synthetic B box (1-74-mer), or full length HMGB1, but the stimulatory effects were specific because the synthetic 20-mers for amino acid fragments containing 16-25, 30-49, 45-64, or 60-74 of the HMGB1 B box did not induce TNF release. These results are direct evidence that the macrophage stimulating activity of the B box specifically maps to the first 20 amino acids of the HMGB B box domain of SEQ ID NO:20). This B box fragment can be used in the same manner as a polypeptide encoding a full length B box polypeptide, for example, to stimulate release of a proinflammatory cytokine, or to treat a condition in a patient characterized by activation of an inflammatory cytokine cascade.



Example 5: HMGB1 A Box Protein Antagonizes HMGB1 Induced Cytokine Activity in a Dose Dependent Manner

Weak agonists are by definition antagonists. Since the HMGB1 A box only weakly induced TNF production, as shown in FIG. 1, the ability of HMGB1 A box to act as an antagonist of HMGB1 activity was evaluated. This study was carried out as follows. Sub-confluent RAW 264.7 cells in 24-well dishes were treated with HMGB1 (1 µg/ml) and 0, 5, 10, or 25 µg/ml of A box for 16 hours in Opti-MEM I medium in the presence of polymyxin B (100 units/ml). The TNF-stimulating activity (assayed using the L929 cytotoxicity assay described herein) in the sample receiving no A box was expressed as 100%, and the inhibition by A box was expressed as percent of HMGB1 alone. The results of the effect of A box on TNF release from RAW 264.7 cells is shown in FIG. 4A. As shown in FIG. 4A, the A box dose-dependently inhibited HMGB1 induced TNF release with an apparent EC<sub>50</sub> of approximately 7.5 µg/ml. Data in FIG. 4A are presented as mean ± SD (n= 2-3 independent experiments).

Example 6: HMGB1 A Box Protein Inhibits Full Length HMGB1 and HMGB1 B Box Cytokine Activity

Antagonism of full length HMGB1 activity by HMGB1 A box or GST tag (vector control) was also determined by measuring TNF release from RAW 264.7 macrophage cultures stimulated by co-addition of A box with full length HMGB1. RAW 264.7 macrophage cells (ATCC) were seeded into 24-well tissue culture plates and used at 90% confluence. The cells were treated with HMGB1, and/or A boxes as indicated for 16 hours in Optimum I medium (Life Technologies, Grand Island, NY) in the presence of polymyxin B (100 units/ml, Sigma, St. Louis, MO) and supernatants were collected for TNF measurement (mouse ELISA kit from R&D System Inc, Minneapolis, MN). TNF-inducing activity was expressed as a percentage of the activity achieved with HMGB1 alone. The results of these studies are shown in FIG. 4B. FIG. 4B is a histogram of the effect of HMGB1 (HMG-1), alone, A box alone, Vector (control) alone, HMGB1 in combination with A box, and HMGB1 in combination with

vector. As shown in FIG. 4B, HMGB1 A box significantly attenuated the TNF stimulating activity of full length HMGB1.

#### Example 7: HMGB1 A Box Protein Inhibits HMGB1 Cytokine Activity by Binding to It

To determine whether the HMGB1 A box acts as an antagonist by displacing  
5 HMGB1 binding,  $^{125}\text{I}$ -labeled-HMGB1 was added to macrophage cultures and binding was measured at 4°C after 2 hours. Binding assays in RAW 264.7 cells were performed as described herein.  $^{125}\text{I}$ -HMGB1 binding was measured in RAW 264.7 cells plated in 24-well dishes for the times indicated in FIG. 5A. Specific binding shown equals total cell-associated  $^{125}\text{I}$ -HMGB1 (CPM/well) minus cell associated CPM/well in the presence  
10 of 5,000 fold molar excess of unlabeled HMGB1. FIG. 5A is a graph of the binding of  $^{125}\text{I}$ -HMGB1 over time. As shown in FIG. 5A, HMGB1 exhibited saturable first order binding kinetics. The specificity of binding was assessed as described in Example 1.

In addition,  $^{125}\text{I}$ -HMG-1 binding was measured in RAW 264.7 cells plated on 24-well dishes and incubated with  $^{125}\text{I}$  HMGB1 alone or in the presence of unlabeled  
15 HMGB1 or A box. The results of this binding assay are shown in FIG. 5B. Data represents mean  $\pm$  SEM from 3 separate experiments. FIG. 5B is a histogram of the cell surface binding of  $^{125}\text{I}$ -HMGB1 in the absence of unlabeled HMGB1 or HMGB1 A box, or in the presence of 5,000 molar excess of unlabeled HMGB1 or HMGB1 A box, measured as a percent of the total CPM/well. In FIG. 5B, "Total" equals counts per  
20 minutes (CPM)/well of cell associated  $^{125}\text{I}$ -HMGB1 in the absence of unlabeled HMGB1 or A box for 2 hours at 4°C. "HMGB1" or "A box" equals CPM/well of cell-associated  $^{125}\text{I}$ -HMGB1 in the presence of 5,000 molar excess of unlabeled HMGB1 or unlabeled A box. The data are expressed as the percent of total counts obtained in the absence of unlabeled HMGB1 proteins (2,382,179 CPM/well). These results indicate that the  
25 HMGB1 A box is a competitive antagonist of HMGB1 activity *in vitro* and inhibits the TNF-stimulating activity of HMGB1.

Example 8: Inhibition of Full Length HMGB1 and HMGB1 B Box Cytokine Activity by Anti-B Box Polyclonal Antibodies.

The ability of antibodies directed against the HMGB1 B box to modulated the effect of full length or HMGB1 B box was also assessed. Affinity purified antibodies  
5 directed against the HMGB1 B box (B box antibodies) were generated as described herein and using standard techniques. To assay the effect of the antibodies on HMGB1-induced or HMGB1 B box-induced TNF release from RAW 264.7 cells, sub-confluent RAW 264.7 cells in 24-well dishes were treated with HMG-1 (1  $\mu$ g/ml) or HMGB1 B box (10  $\mu$ g/ml) for 10 hours with or without anti-B box antibody (25  $\mu$ g/ml or 100  $\mu$ g/ml  
10 antigen affinity purified, Cocalico Biologicals, Inc., Reamstown, PA) or non-immune IgG (25  $\mu$ g/ml or 100  $\mu$ g/ml; Sigma) added. TNF release from the RAW 264.7 cells was measured using the L929 cytotoxicity assay as described herein. The results of this study are shown in FIG. 6, which is a histogram of TNF released by RAW 264.7 cells administered nothing, 1  $\mu$ g/ml of HMGB1, 1  $\mu$ g/ml of HMGB1 plus 25  $\mu$ g/ml of anti-B  
15 box antibody, 1  $\mu$ g/ml of HMGB1 plus 25  $\mu$ g/ml of IgG (control), 10  $\mu$ g/ml of B-box, 10  $\mu$ g/ml of B-box plus 100  $\mu$ g/ml of anti-B box antibody or 10  $\mu$ g/ml of B-box plus 100  $\mu$ g/ml of IgG (control). The amount of TNF released from the cells induced by HMGB1 alone (without addition of B box antibodies) was set as 100%, and the data shown in FIG. 6 are the results of 3 independent experiments. As shown in FIG. 6, affinity  
20 purified antibodies directed against the HMGB1 B box significantly inhibited TNF release induced by either full length HMGB1 or the HMGB1 B box. These results indicate that such an antibody can be used to modulate HMGB1 function.

Example 9: HMGB1 B Box Protein is Toxic to D-galactosamine-sensitized Balb/c Mice

To investigate whether the HMGB1 B box has cytokine activity *in vivo*, we  
25 administered HMGB1 B box protein to unanesthetized Balb/c mice sensitized with D-galactosamine (D-gal), a model that is widely used to study cytokine toxicity (Galanos et al., supra). Briefly, mice (20-25 grams, male, Harlan Sprague-Dawley, Indianapolis, IN) were intraperitoneally injected with D-gal (20 mg) (Sigma, St. Louis, Missouri) and B

box (0.1 mg/ml/mouse or 1 mg/ml/mouse) or GST tag (vector; 0.1 mg/ml/mouse or 1 mg/ml/mouse), as indicated in Table 1. Survival of the mice was monitored up to 7 days to ensure no late death occurred. The results of this study are shown in Table 1.

Table 1: Toxicity of HMGB1 B box on D-galactosamine-sensitized Balb/c Mice

	Treatment	Alive/total
5 Control	-	10/10
Vector	0.1 mg/mouse	2/2
	1 mg/mouse	3/3
B box	0.1 mg/mouse	6/6
	1 mg/mouse	2/8*

\*P<0.01 versus vector alone as tested by Fisher's Exact Test

The results of this study showed that the HMGB1 B box was lethal to D-galactosamine-sensitized mice in a dose-dependent manner. In all instances in which death occurred, it occurred within 12 hours. Lethality was not observed in mice treated with comparable preparations of the purified GST vector protein devoid of B box.

#### Example 10: Histology of D-galactosamine-sensitized Balb/c Mice or C3H/HeJ Mice Administered HMGB1 B Box Protein

To further assess the lethality of the HMGB1 B box protein *in vivo* the HMGB1 B box was again administered to D-galactosamine-sensitized Balb/c mice. Mice (3 per group) received D-gal (20 mg/mouse) plus B box or vector (1 mg/mouse) intraperitoneally for 7 hours and were then sacrificed by decapitation. Blood was collected, and organs (liver, heart, kidney and lung) were harvested and fixed in 10% formaldehyde. Tissue sections were prepared with hematoxylin and eosin staining for histological evaluation (Criterion Inc., Vancouver, Canada). The results of these studies are shown in FIGS. 7A-7J, which are scanned images of hematoxylin and eosin stained

kidney sections (FIG. 7A), myocardium sections (FIG. 7C), lung sections (FIG. 7E), and liver sections (FIGS. 7G and 7I) obtained from an untreated mouse and kidney sections (FIG. 7B), myocardium sections (FIG. 7D), lung sections (FIG. 7F), and liver sections (FIGS. 7H and 7J) obtained from mice treated with the HMGB1 B box. Compared to the control mice, B box treatment caused no abnormality in kidneys (FIGS. 7A and 7B) and lungs (FIGS. 7E and 7F). The mice had some ischemic changes and loss of cross striation in myocardial fibers in the heart (FIGS. 7C and 7D as indicated by the arrow in FIG. 7D). Liver showed most of the damage by the B box as illustrated by active hepatitis (FIGS. 7G-7J). In FIG. 7J, hepatocyte dropouts are seen surrounded by accumulated polymorphonuclear leukocytes. The arrows in FIG. 7J point to the sites of polymorphonuclear accumulation (dotted) or apoptotic hepatocytes (solid). Administration of HMGB1 B box *in vivo* also stimulated significantly increased serum levels of IL-6 (315+93 vs. 20+7 pg/ml, B box vs. control,  $p<0.05$ ) and IL-1 $\beta$  (15+3 vs. 4+1 pg/ml, B box vs. control,  $p<0.05$ ).

Administration of B box protein to C3H/HeJ mice (which do not respond to endotoxin) was also lethal, indicating that HMGB1 B box is lethal in the absence of LPS signal transduction. Hematoxylin and eosin stained sections of lung and kidney collected 8 hours after administration of B box revealed no abnormal morphologic changes. Examination of sections from the heart however, revealed evidence of ischemia with loss of cross striation associated with amorphous pink cytoplasm in myocardial fibers. Sections from liver showed mild acute inflammatory responses, with some hepatocyte dropout and apoptosis, and occasional polymorphonuclear leukocytes. These specific pathological changes were comparable to those observed after administration of full length HMGB1 and confirm that the B box alone can recapitulate the lethal pathological response to HMGB1 *in vivo*.

To address whether the TNF-stimulating activity of HMGB1 contributes to the mediation of lethality by B box, we measured lethality in TNF knock-out mice (TNF-KO, Nowak et al., Am. J. Physiol. Regul. Integr. Comp. Physiol. 278: R1202-R1209, 2000) and the wild-type controls (B6x129 strain) sensitized with D-galactosamine (20

mg/mouse) and exposed to B box (1 mg/mouse, injected intraperitoneally). The B box was highly lethal to the wild-type mice (6 dead out of nine exposed) but lethality was not observed in the TNF-KO mice treated with B box (0 dead out of 9 exposed,  $p < 0.05$  v. wild type). Together with the data from the RAW 264.7 macrophage cultures, described  
5 herein, these data now indicate that the B box of HMGB1 confers specific TNF-stimulating cytokine activity.

#### Example 11: HMGB1 Protein Level is Increased in Septic Mice

To examine the role of HMGB1 in sepsis, we established sepsis in mice and measured serum HMGB1 using a quantitative immunoassay described previously (Wang  
10 et al., supra). Mice were subjected to cecal ligation and puncture (CLP), a well characterized model of sepsis caused by perforating a surgically-created cecal diverticulum, that leads to polymicrobial peritonitis and sepsis (Fink and Heard, supra; Wichmann et al., supra; and Remick et al., supra). Serum levels of HMGB1 were then measured (Wang et al., supra). FIG. 8 shows the results of this study in a graph that  
15 illustrates the levels of HMGB1 in mice 0 hours, 8 hours, 18 hours, 24 hours, 48 hours, and 72 hours after subjection to CLP. As shown in FIG. 8, serum HMGB1 levels were not significantly increased for the first eight hours after cecal perforation, then increased significantly after 18 hours (FIG. 8). Increased serum HMGB1 remained at elevated plateau levels for at least 72 hours after CLP, a kinetic profile that is quite similar to the  
20 previously-described, delayed HMGB1 kinetics in endotoxemia (Wang et al., supra). This temporal pattern of HMGB1 release corresponded closely to the development of signs of sepsis in the mice. During the first eight hours after cecal perforation the animals were observed to be mildly ill, with some diminished activity and loss of exploratory behavior. Over the ensuing 18 hours the animals became gravely ill,  
25 huddled together in groups with piloerection, did not seek water or food, and became minimally responsive to external stimuli or being examined by the handler.

#### Example 12: Treatment of Septic Mice with HMGB1 A Box Protein Increases Survival of Mice

To determine whether the HMGB1 A box can inhibit the lethality of HMGB1 during sepsis, mice were subjected to cecal perforation and treated by administration of A box beginning 24 hours after the onset of sepsis. CLP was performed on male Balb/c mice as described herein. Animals were randomly grouped, with 15-25 mice per group. The HMGB1 A box (60 or 600  $\mu\text{g}/\text{mouse}$  each time) or vector (GST tag, 600  $\mu\text{g}/\text{mouse}$ ) alone was administered intraperitoneally twice daily for 3 days beginning 24 hours after CLP. Survival was monitored twice daily for up to 2 weeks to ensure no late death occurred. The results of this study are illustrated in FIG. 9, which is a graph of the effect of vector (GST; control) 60  $\mu\text{g}/\text{mouse}$  or 600  $\mu\text{g}/\text{mouse}$  on survival over time (\* $P < 0.03$  vs. control as tested by Fisher's exact test). As shown in FIG. 9, administration of the HMGB1 A box significantly rescued mice from the lethal effects of sepsis, and improved survival from 28% in the animals treated with protein purified from the vector protein (GST) devoid of the A box, to 68% in animals receiving A box ( $P < 0.03$  by Fischer's exact test). The rescuing effects of the HMGB1 A box in this sepsis model were A box dose-dependent; animals treated with 600  $\mu\text{g}/\text{mouse}$  of A box were observed to be significantly more alert, active, and to resume feeding behavior as compared to either control animals treated with vector-derived preparations, or to animals treated with only 60  $\mu\text{g}$  A box. The latter animals remained gravely ill, with depressed activity and feeding for several days, and most died.

#### Example 13: Treatment of Septic Mice with Anti-HMGB1 Antibody Increases Survival of Mice

Passive immunization of critically ill septic mice with anti-HMGB1 antibodies was also assessed. In this study, male Balb/c mice (20-25 gm) were subjected to CLP, as described herein. Affinity purified anti-HMGB1 B box polyclonal antibody or rabbit IgG (as control) was administered at 600  $\mu\text{g}/\text{mouse}$  beginning 24 hours after the surgery, and twice daily for 3 days. Survival was monitored for 2 weeks. The results of this

study are shown in FIG. 10A, which is a graph of the survival of septic mice treated with either a control antibody or an anti-HMGB1 antibody. The results show that anti-HMGB1 antibodies administered to the mice 24 hours after the onset of cecal perforation significantly rescued animals from death as compared to administration of non-immune antibodies (p<0.02 by Fisher's exact test). Within 12 hours after administration of anti-HMGB1 antibodies, treated animals showed increased activity and responsiveness as compared to controls receiving non-immune antibodies. Whereas animals treated with non-immune antibodies remained huddled, ill kempt, and inactive, the treated animals improved significantly and within 48 hours resumed normal feeding behavior. Anti-HMGB1 antibodies did not suppress bacterial proliferation in this model, because we observed comparable bacterial counts (CFU, the aerobic colony forming units) from spleen 31 hours after CLP in the treated animals as compared to animals receiving irrelevant antibodies (control bacteria counts =  $3.5 \pm 0.9 \times 10^4$  CFU/g; n=7). Animals were monitored for up to 2 weeks afterwards, and late deaths were not observed, indicating that treatment with anti-HMGB1 conferred complete rescue from lethal sepsis, and did not merely delay death.

To our knowledge, no other specific cytokine-directed therapeutic is as effective when administered so late after the onset of sepsis. By comparison, administration of anti-TNF actually increases mortality in this model, and anti-MIF antibodies are ineffective if administered more than 8 hours after cecal perforation (Remick et al, supra; and Calandra et al., *Nature Med.* 6:164-170, 2000). These data demonstrate that HMGB1 can be targeted as late as 24 hours after cecal perforation in order to rescue lethal cases of established sepsis.

In another example of the rescue of endotoxemic mice using anti-B box antibodies, anti-HMGB1 B box antibodies were evaluated for their ability to rescue LPS-induced septic mice. Male Balb/c mice (20-25 gm, 26 per group) were treated with an LD75 dose of LPS (15 mg/kg) injected intraperitoneally (IP). Anti-HMGB1 B box or non-immune rabbit serum (0.3 ml per mouse each time, IP) was given at time 0, +12 hours and +24 hours after LPS administration. Survival of mice was evaluated over



time. The results of this study are shown in FIG. 10B, which is a graph of the survival of septic mice administered anti-HMGB1 B box antibodies or non-immune serum. As shown in FIG. 10B, anti-HMGB1 B box antibodies improved survival of the septic mice.

#### Example 14: Inhibition of HMGB1 Signaling Pathway Using an Anti-RAGE Antibody

5        Previous data implicated RAGE as an HMGB1 receptor that can mediate neurite outgrowth during brain development and migration of smooth muscle cells in wound healing (Hori et al. J. Biol. chem. 270:25752-25761, 1995; Merenmies et al. J. Biol. Chem. 266:16722-16729, 1991; and Degryse et al., J. Cell Biol. 152:1197-1206, 2001). We measured TNF release in RAW 264.7 cultures stimulated with HMGB1 (1 µg/ml),  
10 LPS (0.1 µg/ml), or HMGB1 B box (1 µg/ml) in the presence of anti-RAGE antibody (25 µg/ml) or non-immune IgG (25 µg/ml). Briefly, the cells were seeded into 24-well tissue culture plates and used at 90% confluence. LPS (*E. coli* 0111:B4, Sigma, St. Louis, MO) was sonicated for 20 minutes before use. Cells were treated with HMGB1 (HMG-1; 1 µg/ml), LPS (0.1 µg/ml), or HMGB1 B box (B Box; 1 µg/ml) in the  
15 presence of anti-RAGE antibody (25 µg/ml) or non-immune IgG (25 µg/ml), as indicated in FIG. 11A, for 16 hours in serum-free Opti-MEM I medium (Life Technologies) and supernatants were collected for TNF measurement using the L929 cytotoxicity assay described herein. IgG purified polyclonal anti-RAGE antibody (Catalog No. sc-8230, N-16, Santa Cruz Biotech, Inc., Santa Cruz, CA) was dialyzed  
20 extensively against PBS before use. The results of this study are shown in FIG. 11A, which is a histogram of the effects of HMGB1, LPS, or HMGB1 B box in the presence of anti-RAGE antibodies or non-immune IgG (control) on TNF release from RAW 264.7 cells. As shown in FIG. 11A, compared to non-immune IgG, anti-RAGE antibody significantly inhibited HMGB1 B box-induced TNF release. This suppression was  
25 specific, because anti-RAGE did not significantly inhibit LPS-stimulated TNF release. Notably, the maximum inhibitory effect of anti-RAGE decreased HMG-1 signaling by only 40%, suggesting that other signal transduction pathways may participate in HMGB1 signaling.

To examine the effects of HMGB1 or HMGB1 B box on the NF- $\kappa$ B-dependent ELAM promoter, the following experiment was carried out. RAW 264.7 macrophages were transiently co-transfected with an expression plasmid encoding a murine MyD 88-dominant-negative (DN) mutant (corresponding to amino acids 146-296), or empty  
5 vector, plus a luciferase reporter plasmid under the control of the NF- $\kappa$ B-dependent ELAM promoter, as described by Means et al. (J. Immunol. 166:4074-4082, 2001). A portion of the cells were then stimulated with full-length HMGB1 (100 ng/ml), or purified HMGB1 B box (10  $\mu$ g/ml), for 5 hours. Cells were then harvested and luciferase activity was measured, using standard methods. All transfections were  
10 performed in triplicate, repeated at least three times, and a single representative experiment is shown in FIG. 11B. As shown in FIG. 11B, HMGB1 stimulated luciferase activity in samples that were not co-transfected with the MyD 88 dominant negative, and the level of stimulation was decreased in samples that were co-transfected with the MyD 88 dominant negative. This effect was also observed in samples administered HMGB B  
15 box.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.